ONTOGENIC RESISTANCE TO UNCINULA NECATOR IN DEVELOPING BERRIES OF GRAPEVINE (VITIS SPP.): USING GERMPLASM DIVERSITY TO STUDY THE MECHANISM

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ONTOGENIC RESISTANCE TO UNCINULA NECATOR IN DEVELOPING BERRIES OF GRAPEVINE (VITIS SPP.): USING GERMPLASM DIVERSITY TO STUDY THE MECHANISM

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Berries of several grapevine (*Vitis spp.*) species develop resistance to the powdery mildew fungus *Uncinula necator*, as they age, becoming resistant within approximately two weeks after bloom (ontogenic resistance). Ontogenic resistance is of interest to uncover new avenues of disease management and to expand our knowledge of fruit development. Several studies were developed to advance this goal. A screen of wild *Vitis* spp. and interspecific hybrids was conducted to identify and describe phenotypic variation. This project was successful in both expanding our understanding of the population dynamics of ontogenic resistance and in identifying genotypes with suitable variation for a hypothesis-driven examination of berry cuticle development and for a discovery-science oriented investigation of protein expression of berry peels correlated to the gain of resistance. The protein expression profiling was successful in identifying 142 proteins differentially expressed during early berry development from four selected genotypes of *Vitis*, including 45 whose expression most closely associated with the gain of resistance. Overall, these proteins are enriched for biological functions involved in metabolism, cell regulation and proteolysis. Fifty-five sequences in the newly released *V. vinifera* genome were also newly annotated for function, further improving this emerging genetic resource. The studies into the development of the berry cuticle as it relates to ontogenic resistance were successful in showing that both epicuticular wax chemistry as well as the surface

topology are possibly involved in the gain of resistance. These studies have propelled our understanding of this potentially useful form of host resistance, which could eventually lead to new practices of disease control in the vineyard resulting in better disease control and less environmental impact.

BIOGRAPHICAL SKETCH

Christopher was born to James and Joan Gee in Lansing, Michigan, and raised outside the small town of Williamston. Christopher was always interested in the natural world around him; he grew up receiving many lessons in plants and animals, whether it was digging in the garden, catching bugs and frogs in the woods, or just watching the animals and plants around him.

After graduating from Williamston High School, Christopher attended Michigan State University in East Lansing, Michigan where he majored in botany and plant pathology, with a focus on plant pathology and mycology. During his time at MSU, Christopher learned a great deal about plants, including work in botanical garden management, plant physiology, and horticulture. A lover of the kingdom Plantae, he sought out every opportunity to learn as much as possible about its members.

Christopher began his Ph.D. studies in plant pathology at Cornell University in August 2001 in Ithaca, NY. Moving to Geneva, NY in June, 2004, Christopher completed his doctorate in January, 2009 in the laboratory of Dr. Lance Cadle-Davidson, working on the molecular basis of ontogenic resistance in grapevine.

This dissertation is dedicated to my parents, who deserve very special credit. Whether they know it or not, it is because of them that I became so fascinated with nature.

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From the beginning, my family has been the utmost supportive, even without any understanding of what I really do. My parents and my brother were always there to lend me support any way they could, whether it would be to listen to me vent, or just by giving me encouragement to just finish this book and get a job. I have learned that backup is crucial when taking on this much work, and they were the best backup a boy could ask for.

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For anyone who reads beyond this page, I wish you luck. The majority of the content for this book was hashed out in the middle of the night. There is a very real chance it only makes sense to me.

~Chris (a.k.a. the PhDJ)

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LIST OF ABBREVIATIONS

ACN – Acetonitrile

BSTFA – N,O-Bis(trimethylsilyl)trifluoroacetamide

CHAPS – 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CR – Constitutively resistant

DIGE – Difference Gel Electrophoresis

DTT – Dithiothreitol

GC-FID – Gas chromatography-Flame ionization detection

GC-MS – Gas chromatography-Mass spectrometry

HPLC – High-performance liquid chromatography

IEF – Isoelectric focusing

IPG – Immobilized pH gradient

LC-ESI-MS/MS – Liquid chromotograph – Electrospray ionization – Tandem mass spectrometry

MALDI – Matrix-assisted laser desorption/ionization

MS/MS – Tandem mass spectrometry

NanoLC – Nano-bore liquid chromatography

NS – Non-significant resistance pattern

OR – Ontogenic resistance

SB 3-10 – (n-Decyl-N,N-dimethyl-c-ammonio-1-porpanesulfonate)

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SU – Persistently susceptible

TBP – Tributyl phosphine

TCEP – Tris(2-carboxyethyl)phosphine

TMS – trimethylchlorosilane

Tris – Trishydroxymethylaminomethane

INTRODUCTION

A complex interplay exists between host and pathogen. The host resists the attacks of the pathogen, which in turn relies on an ability to infect, colonize and extract resources from the host. In this relationship the relative ability of each participant to carry out the desired activity can change in effectiveness due to environmental or temporal influences. Plant hosts can experience changes in susceptibility within or between growing seasons. Many such examples exist in important agricultural pathosystems, and are of interest in the effort to improve production of important food supplies.

In developmentally-regulated resistance, the temporal change of host susceptibility is linked to the state of biological development. This results most frequently in aging or mature tissue exhibiting a quantitative reduction in susceptibility to a pathogen, regardless of environmental or pathogen population dynamics. Many names have been applied to this type of resistance, including age-related resistance, adult-plant resistance and ontogenic resistance [3, 12, 29, 36]. Broadly, developmentally regulated resistance has been observed in several pathosystems, such as those including bacteria, fungi, nematodes, insects and Oomycetes [2, 6, 14, 19, 22, 23, 29, 31], and ranges in effectiveness from conferring minor quantitative levels to near-immunity. Multiple mechanisms have been suggested for developmentally-regulated resistances, such as senescence-induced resistance, flowering-induced resistance, and others that might include multiple pathways simultaneously [29].

Powdery mildew pathosystems exhibiting developmentally-regulated gain of resistance have been genetically characterized in several pathosystems including wheat [16, 17, 28, 38], barley [1, 21, 34], apple [5, 37] and soybean [15, 25]. Across

these powdery mildew pathosystems, ontogenic resistance is conferred by various genetic mechanisms, from single gene resistance to quantitative or additive effects controlled by quantitative trait loci (QTL). Notably, in the aforementioned examples, resistance was investigated on foliage of the host, and not upon fruit, which typically exhibit a more protracted and complex developmental process than foliage. Among these pathosystems, the system studied most in-depth is powdery mildew (*Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* Em. Marchal, syn. *Erysiphe graminis* f. sp. *tritici*) on wheat (*Triticum aestivum*).

The most common form of powdery mildew resistance used in wheat breeding programs has been the deployment of race-specific resistance genes that are expressed throughout vegetative development and confer a hypersensitive resistance [24, 39]. This type of resistance has shown a lack of durability once the pathogen population shifts to races virulent on host plants containing the resistance gene [20, 24, 32, 35]. In contrast, adult-plant resistance under the control of single or multiple QTLs in wheat has been found to confer durable and broad spectrum control [20, 24, 26]. This particular developmentally-regulated resistance occurs infrequently in accessions of *T. aestivum* [38] and does not confer an immunity response, but rather a slowing of pathogen progression termed slow mildewing or partial resistance [18, 35].

Grapevine (*Vitis spp.*) is one of the most important fruit crops in the world, with 7.4 million hectares of production worldwide yielding 68 million metric tons of fruit annually [27]. Grapes are grown for use in the production of wine and juice, for raisins, and for table consumption. A major impediment to this production effort is the powdery mildew fungus (*Uncinula necator* (Schwein.) Burrill, syn. *Erysiphe necator*), which occurs in all major grape growing regions and can be an extremely

damaging pathogen [30].

Powdery mildew can colonize the shoots, leaves and fruit, defoliate vines and render fruit unusable [10]. The window of berry susceptibility lasts approximately two weeks following anthesis [8, 11-13, 36]. The susceptibility is maximal at bloom and decreases rapidly until the tissue becomes nearly immune to new infections while halting the expansion of existing colonies [8, 11, 12, 36]. Within a cluster, there may be variation in the timing and magnitude of ontogenic resistance between different tissue types (*e.g.* berries, pedicel and rachis) in ontogenic resistance [11].

Expression of ontogenic resistance in grape berries neither inhibits the attachment or germination of conidia, nor the formation of appressorium on the berry surface [7, 9]. It does however decrease the density and fecundity of mildew colonies [9]. Ontogenic resistance operates by stopping successful entry of the fungal penetration peg before the formation of a visible penetration pore,, thereby thwarting the infection attempt [9]. Ontogenic resistance was not a consequence of thickening of the cuticle or anticlinal cell wall, accumulation of antimicrobial polyphenols, formation of papillae beneath the appressorium or activity of some known plant resistance genes [7]. These results suggested the formation of a barrier, chemical or physical, at or near the berry surface, or the rapid accumulation of anti-fungal compounds in resistant berries during the first few hours of infection [7].

In the current study, the initial step to expand our understanding of ontogenic resistance was a disease resistance screen of 79 varied genotypes covering six species and several interspecific hybrids of *Vitis*, the published results of which are presented in Chapter 1 [13]. The primary purpose of this screen was to identify new sources of

phenotypic variation in grapevine, since the variation identified previously [8, 11, 12, 36] was not considered sufficient for planned molecular studies.

The population screened in this study was highly enriched for North American grapevine species, a known source for high levels of resistance and tolerance to powdery mildew. This was confirmed as 63% of the total population exhibited a constitutive resistance in the fruit, completely resistant to inoculations happening as early as four days post bloom. Ontogenic resistance was expressed overwhelmingly in the remaining genotypes. This group had a majority of genotypes possessing some *V. vinifera* in their pedigree, demonstrated by the presence of hermaphroditic flowers. Some members from North American species also had an ontogenic resistance phenotype, although with an overall with a lower initial quantitative susceptibility. In select genotypes, ontogenic resistance was confirmed to be a penetrative resistance, as previously identified [13]. Most genotypes had tissue-specific resistance profiles, primarily with resistant berries and susceptible rachises and/or pedicels.

An exciting find was the identification and characterization of the first genotype found to remain susceptible during development, beyond one month post-bloom in vineyard experiments and beyond veraison when using *in vitro* assays. This wild-collected *V. rupestris* exhibits a novel protracted period of susceptibility in the clusters, but otherwise appears to be a normally developing vine. This genotype provided phenotypic variation in ontogenic resistance for future studies.

In an effort to generate new hypotheses regarding the molecular basis of ontogenic resistance, a comparative proteomics project was conducted utilizing two-dimensional polyacrylamide gels, Difference In-Gel Electrophoresis and an analysis strategy

involving *in silico* bulking of biological samples based on phenotype. This study, communicated in Chapter 2, examines protein expression in the berry skins of three *Vitis* interspecific hybrids which exhibit a typical ontogenic resistance and of the *V. rupestris* that remains susceptible.

The bulking method, which was adapted from a similar study conducted in non-related accessions of *Oryza sativa* [33], was aimed at uncoupling the gain of resistance from the concomitant biological activity unrelated to the gain of resistance. To this end, the bulk composed of susceptible samples contained the young (x to y days post bloom) and older samples (m and n days post bloom) of the susceptible *V. rupestris* and only the young, susceptible samples from the other genotypes. Conversely, the resistant bulk contained only older samples (x and y days post bloom) from the three ontogenic resistant hybrids. This experimental design, with older samples in resistant and susceptible bulks, aimed to achieve this uncoupling and enrich for protein expression related to the gain of resistance.

The study was successful at identifying a set of proteins whose expression correlates to the gain of resistance in grape berries, proteins whose expression is conserved across the selected genotypes and those which show a genotype-specific pattern of expression. There is a strong pattern of metabolic, photosynthetic and regulatory proteins showing differential expression during development, especially in those spots with the most correlated significant expression fold-change between susceptible and resistant tissue. This would suggest that the processes leading to ontogenic resistance lie in normal berry development. This hypothesis is further supported by the lack of proteins being identified which are known to be involved in disease and stress response.

A role for the cuticle was strongly suggested [7, 9] in the research which provided the foundation for research in grapevine ontogenic resistance. The aforementioned demonstration of the inability for powdery mildew conidia to penetrate ontogenically resistant berries, along with well documented evidence for extensive chemical and physical changes during berry cuticle development [4], was studied and reported in Chapter 3. Once again relying on genotypes selected from the phenotypic screen, but this time also including a genotype expressing constitutive resistance, a study of the chemical development of the grape berry waxes and topological development of the berry surface was conducted.

A consistent pattern of chemical composition was found between genotypes through early berry development. The cuticle composition was dominated by the presence of oleanolic acid, a triterpanoid. Minor constituents consisted of various proportions of *n*-alkanes, long-chain fatty acids, aldehydes and primary fatty-alcohols. Some genotypic variation in the chemical development was noticed, including possible correlations between chemistry and resistance.

In contrast to the relatively consistent pattern in cuticle chemistry, extensive changes were seen in cuticle topology. While all genotypes exhibited a pattern of change in surface architecture, genotypic differences in cuticle were clear at 28 days post bloom. This difference correlated to the gain of resistance, with the susceptible genotype possessing a cuticle distinct from other representatives of genus. All three genotypes, regardless of phenotype, exhibited the same structural features at the beginning of berry development. This means that the earliest time point in the constitutively resistant genotype had a cuticle surface topology as the susceptible developmental stage of the genotype expressing ontogenic resistance and the genotype which never

gains resistance.

These studies have greatly expanded our understanding of the presence and dynamics of ontogenic resistance in grape berries, including the identification of new sources of genotypic variation which will provide the basis for future studies. New ideas are being formed to further explore this type of host resistance. Generation of new genetic lines using genotypes identified during this project can be used to further explore areas of interest and to fine-tune additional studies in cuticle development.

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Chapter 1

Ontogenic resistance to *Uncinula necator* varies by genotype and tissue-type in a diverse collection of *Vitis spp.**

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ABSTRACT

Berries of grapevine (*Vitis spp.*) have a limited window of susceptibility to *Uncinula necator*, the causal agent of grapevine powdery mildew, until the onset of ontogenic resistance approximately 2 to 3 weeks post-bloom. This phenomenon has been demonstrated in several cultivars of *V. vinifera* and in *V. labruscana* 'Concord', which all exhibited a similar duration of susceptibility. To identify genetic variation for ontogenic resistance, we screened a diverse collection of *Vitis* species and interspecific hybrids maintained in the USDA-ARS cold-hardy *Vitis* germplasm collection in Geneva, NY. Of the 79 genotypes whose fruit clusters were screened for susceptibility to powdery mildew under field conditions, 50 exhibited a high level of basal resistance to powdery mildew and did not develop more than trace levels of disease when inoculated, irrespective of the stage of berry development at inoculation. Twenty-four genotypes exhibited a significant gain of resistance as berries aged. This ontogenic resistance was conserved across four species and several interspecific hybrids of *Vitis spp.*, though the timing of the onset of ontogenic resistance varied by genotype. The mechanism of ontogenic resistance was examined for four genotypes. Similar to previous studies, ontogenic resistance greatly reduced the incidence of successful penetration. Despite the broad conservation of ontogenic resistance across species, one genotype (*V. rupestris* 'R-65-44') remained susceptible past the onset of ripening, over one month later than reported previously for *V. vinifera* and *V. labruscana*. Variation in the resistance phenotype was observed among the rachis, pedicels and berries within clusters of the majority of genotypes studied. The genetic variation in ontogenic resistance, in particular the discovery of a genotype in which berries remain susceptible as they mature, will facilitate further study of the inheritance and molecular basis of ontogenic resistance.

INTRODUCTION

Interaction between pathogens and their plant hosts is dynamic, with susceptibility changing as whole plants, organs, and specific tissues age. The principal terms used to describe this process include age-related resistance, adult plant resistance, and ontogenic resistance [3, 11, 16, 21]. Broadly defined, such developmentally-regulated resistance is exhibited toward many plant pests, including fungi, viruses, bacteria, nematodes, oomycetes and insects, and ranges from partial resistance to immunity [2, 6, 12-16, 18].

In grapevine (*Vitis spp.*), berries of *V. vinifera* and the interspecific *V. vinifera* hybrid *V. labruscana* 'Concord' [1] are highly susceptible to infection by grapevine powdery mildew (*Uncinula necator* (Schwein.) Burrill, syn. *Erysiphe necator*) for a period beginning with bloom and lasting approximately two to three weeks [8, 10, 11, 21]. After this time period, susceptibility rapidly decreases until the berries develop ontogenic resistance, characterized by near-immunity to subsequent infection or to further colonization by existing infections [10, 11]. The cause of this resistance remains elusive but is attributed neither to factors commonly involved in age-related resistances, such as thickening of the berry cuticle or the anticlinal cell wall, nor to the formation of papillae or typical PR gene products (*e.g.* thaumatin and chitinase) [7]. This gain of resistance is expressed differently in separate tissues of the cluster (*i.e.* rachis, berries) [10], which may be due to differences in timing of the onset of resistance, or to a different mechanism.

In previous studies, several genotypes of *Vitis* exhibited a gain of resistance that occurred on a similar time-frame and apparently halted the pathogen attack at the

cuticle, immediately prior to penetration [8, 10, 11, 21]. The lack of variation hindered studies of the genetic basis of ontogenic resistance, as it was impossible to separate possible resistance factors from factors related to normal berry development. Further progress in understanding ontogenic resistance would be greatly aided by discovery of genetic variation in the trait. Thus, we began a more comprehensive search for variation in ontogenic resistance within *Vitis spp.*, in which we assessed the temporal distribution of resistance to *U. necator* among a genetically diverse collection of 79 host genotypes. These genotypes included six *Vitis* species and several *Vitis* interspecific hybrids maintained in the USDA-ARS cold-hardy *Vitis* germplasm collection. Tissue specificity of ontogenic resistance to *U. necator* was also quantified and characterized within this collection.

MATERIALS AND METHODS

Vineyard and treatments. Twenty-year-old field-grown vines at the USDA-ARS cold-hardy *Vitis* germplasm collection located in Geneva, New York were used in these experiments. Seventy-nine genotypes of pistillate and hermaphroditic flowered vines were selected for evaluation for the gain of ontogenic resistance. Each genotype in the germplasm collection is represented by two adjacent, replicate, own-rooted vines planted on 1.8 m centers using a three-wire trellis, cane-pruned and trained to the Umbrella Kniffin system. Each genotype was tracked using the unique plant introduction (PI) number taken from the USDA-ARS Germplasm Resources Information Network (GRIN) database [23]. To limit contamination of clusters with natural powdery mildew prior to inoculation, the vineyard was sprayed every 14 days using a typical commercial spray program consisting of locally systemic and contact fungicides [10]. To ensure that clusters selected for inoculation were protected from

chemical sprays, clusters were covered with plastic bags prior to fungicide application as previously described [10].

Primary basal clusters were randomly assigned to inoculations targeted at 4, 8, 15, 21 or 28 days post bloom (DPB), unless otherwise stated. Each treatment consisted of two replicate clusters on each of the two adjacent replicate vines. Bloom was defined as the point at which the calyptra of 50% of flowers within a cluster had dehisced. On each vine, a single cluster was protected from fungicide application but left uninoculated to ascertain the natural levels of infection within the experimental vineyard. To limit the numbers of clusters to be inoculated, genotypes with less than 10% severity – defined as the susceptibility threshold – at all time points in 2004 were inoculated at only one early (6 DPB) and one late (27 DPB) timepoint in both 2005 and 2006, to confirm resistance.

Inoculum preparation and cluster inoculations. Conidial inoculum was raised on grapevine seedlings, grown from seeds harvested either from *V. vinifera* 'Riesling' or *V. vinifera* 'Chardonnay'. Germinated seeds were sown in 3-inch pots and grown to the 4^{th} leaf stage in a greenhouse under natural light conditions, then placed in an environment controlled growth chamber set at 25°C with 12 hours of light. In all three years of the study, mixtures of sexually-incompatible isolates were released into the chamber to start an epidemic, which was maintained with the addition of new seedlings.

Heavily mildewed leaves with actively growing colonies were vortexed in 18.2MΩ water (Barnstead E-Pure, Dubuque, IA) with 0.005 % Tween-20 (Sigma-Aldrich, St. Louis, MO) to suspend the conidia. Spore density was corrected to 10^5 conidia/ml

after quantification using a hemacytometer. The spore suspension was sprayed onto clusters until droplet runoff using a handheld atomizer (Preval, PrecisionValve, Yonkers, NY), using care to prevent unintended inoculations. Spore suspensions were discarded and prepared fresh every 30 minutes to promote high spore viability.

Data collection, management, and statistical analysis. Clusters were marked with unique bar codes affixed to slip-on tags hung around the rachis of the cluster prior to bloom. Bar codes were tracked using a Symbol MC50 handheld computer (Motorola, Inc., Holtsville, NY), which used custom written software to interface with the database while in the vineyard. All clusters were tracked for bloom and inoculation dates, as well as disease severity and cluster survival.

Following an incubation of six to nine weeks post inoculation, clusters were assessed macroscopically by visually estimating the percentage of surface covered by powdery mildew using 5% increments. Within a cluster, the rachis, pedicels, and berries were separately rated for disease. The same person (C.T.G.) assessed disease severity in all seasons for all tissues. For berry disease assessment, severity on normally-developing berries was recorded separately from severity on shot berries, which are stunted in growth and development [5]. Cluster age at inoculation was translated from DPB to Growing Degree Days (GDD, base = 10°C) postbloom to account for the year-to-year environmental effects. Daily temperature data were obtained from a weather monitoring station located 2.5 km from the vineyard at the New York State Agricultural Experiment Station in Geneva, NY.

Statistical analysis was carried out using PROC GLM in SAS v.8.0 (SAS Institute, Inc., Cary, NC) using disease severity as the response variable and GDD, field-year and the interaction GDD x field year as predictors. The type-I sums of squares for GDD and GDD x field year from the GLM analysis were summed and analyzed in a nested design to determine their contribution to overall significance. P-values were determined using the FDIST function in Excel 2003 (Microsoft Corporation, Redmond, Washington). The mean slope and intercept of disease severity by GDD for each genotype was determined using the Fit Model module in JMP v.7.0 (SAS Institute, Inc., Cary, NC). The y-intercept of the line of best fit was taken as the maximum severity, or the potential disease severity at time=0. The last susceptible time was calculated by setting disease severity=10%, and using the parameter estimates for the least squares fit (JMP Fit Model module) to solve for GDD.

Genotypes were classified into four phenotypic groups: 1) constitutively resistant (CR), 2) persistently susceptible (SU), 3) ontogenic resistant (OR), and 4) not significant (NS). CR genotypes were always resistant and were therefore defined by having a disease severity of 10% or less on all inoculated clusters. Of the remaining genotypes, which had susceptibility at some point during development, SU genotypes failed to develop resistance and were therefore classified by having a disease severity greater than 10% over the last 20% of the screening period (in GDD), averaged between all years. For the remaining genotypes, categorization of OR required that the statistical analysis described above be statistically significant (α=0.1) (Table 1.1). These three categories are illustrated in Figure 1.1 using specific genotypes as examples. Genotypes not fitting into one of the three above categories were labeled as NS, meaning no significant pattern could be applied. Genotypes which were identified as CR for the berries were not included in the statistical analysis.

Table 1.1. Phenotypic classification of genotypes with susceptible berries, organized by berry phenotype

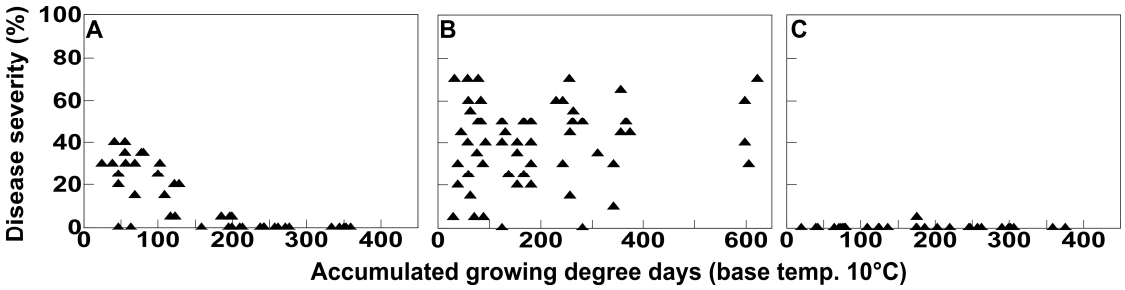
				Berry					
Berry classification[a]	PI[b]	*Vitis* species[c]	Variety[d]	Early severity (%)[e]	P-value[f]	Late severity (%)[g]	Predicted time of <10% severity (GDD)[h]	Rachis Classification[i]	Pedicel Classification[j]
OR	588296	*V. hybrid*	Red Amber (Minn 25)	30.6	<0.001	0.0	171.4	SU	SU
	588313	*V. hybrid*	Carman	25.5	<0.001	0.0	194.8	SU	OR
	588340	*V. hybrid*	unknown	18.9	0.022	5.8	202.7	SU	SU
	588430	*V. hybrid*	Wapanuka	12.4	0.084	8.8	68.2	SU	SU
	588474	*V. hybrid*	NY 66.760.2	22.0	<0.001	6.3	184.7	SU	SU
	588477	*V. hybrid*	NY 65.556.1	31.1	<0.001	1.5	185.1	SU	SU
	588484	*V. hybrid*	NY 65.591.1	39.3	<0.001	5.6	276.9	SU	SU
	588504	*V. hybrid*	NY 65.546.3	35.7	<0.001	1.5	225.3	SU	OR
	588505	*V. hybrid*	NY 65.562.2	11.2	<0.001	0.0	35.2	NS	NS
	588517	*V. hybrid*	Ill 487-1	12.9	0.004	0.0	65.8	SU	SU
	588519	*V. hybrid*	NY 65.575.1	38.1	<0.001	0.0	284.3	SU	SU
	588524	*V. hybrid*	Ill 803-2	20.3	<0.001	0.0	138.1	SU	SU
	588527	*V. hybrid*	NY 65.004.1	18.0	<0.001	0.0	131.9	SU	SU
	588530	*V. hybrid*	NY 65.556.5	29.2	<0.001	2.5	191.7	SU	SU
	588550	*V. hybrid*	Reinohli	12.2	0.004	0.0	42.4	OR	SU
	588564	*V. hybrid*	John Viola	16.5	<0.001	0.0	75.7	OR	OR
	597247	*V. hybrid*	Gaertner	31.7	<0.001	0.0	114.3	OR	OR
	483147	*V. labrusca*	Rem 43-75	11.0	0.040	0.0	21.4	NS	SU
	483163	*V. labrusca*	Rem NE 36	15.2	0.003	0.0	69.1	SU	SU
	588194	*V. labrusca*	Dunkel 1	11.3	0.053	1.3	35.0	NS	SU
	483181	*V. riparia*	Rem NE 22	13.3	0.067	0.6	80.2	NS	SU
	588225	*V. rupestris*	R-65-47	6.9	0.006	0.3	-110.1	SU	SU
	588231	*V. rupestris*	Alphonse de Serres	9.0	0.007	0.0	-30.3	SU	SU
	588401	*V. rupestris*	RU-66-2	14.7	0.054	0.0	83.7	SU	SU
SU	588224	*V. rupestris*	R-65-44	40.2	0.021	36.7	1804.0	SU	SU

Table 1.1 (continued)

NS	588217	*V. cinerea*	B 27	9.7	0.100	0.0	-9.1	SU	SU
	588398	*V. cinerea*	Barrett 1	11.7	0.114	0.0	22.9	SU	NS
	588493	*V. hybrid*	NY 65.586.1	12.3	0.187	2.6	100.8	SU	SU
	588497	*V. hybrid*	NY 65.552.2	14.1	0.299	0.0	77.2	NS	NS

[a.] Classification categories used: OR = ontogenically resistant, SU = persistently susceptible, CR = constitutively resistant, NS = non-significant.
[b.] PI = Plant Introduction number, a unique identifier for each genotype in the GRIN database, where PI is linked with additional information related to that genotype: http://www.ars-grin.gov/npgs/index.html (13).
[c.] Species listed for each genotype, taken from the GRIN database. *Vitis* interspecific hybrids are listed as *V. hybrid*.
[d.] Variety listed for each genotype, taken from the GRIN database. Genotypes with no variety recorded listed as unknown.
[e.] Early disease severity of berries determined by taking the y-intercept value from the best fit equation using the Fit Model module in JMP v.7 (see methods).
[f.] P-value of the F-test for the effect of growing degree days accumulated after bloom and its interaction with year. This calculation uses the Type-I sums of squares from the GLM output of SAS 8.0.
[g.] Late severity averaged over the last 20% of the screening-period across all years.
[h.] GDD at which the berry severity is predicted to fall below 10%, calculated by using the equation for the mean slope for each genotype, as determined using the Fit Model module in JMP v.7 (see methods). For those genotypes with y-intercepts less than 10% severity, this number is negative.
[i.] Classification of the rachis using the same criteria developed for berry data.
[j.] Classification of the pedicel using the same criteria developed for berry data.

Figure 1.1. Graphical representatives of three phenotypic categories used to classify the 79 genotypes screened. All graphs show a composite of three years of data for genotypes: (A) *Vitis hybrid* 'Red Amber' (PI: 588296) showing a typical ontogenic gain of resistance (OR); (B) *V. rupestris* 'R-65-44' (PI: 588224) showing persistent susceptibility (SU) well beyond typical development of ontogenic resistance (note x-axis scale); and (C) *V. hybrid* 'Tom's Favorite' (PI: 483176) showing constitutive resistance (CR). Each filled triangle represents disease severity (y-axis) of one or more replicate clusters inoculated at a calculated number of growing degree days after 50% bloom (x-axis).


Disease severity (%)
100
80
60
40
20
0
A
B
C
0
100
200
300
400
0
200
400
600
0
100
200
300
400
Accumulated growing degree days (base temp. 10°C)

Detached berry preparation and epi-illuminescence microscopy. In 2006 and 2007, berries were collected 50-75 DPB from clusters of selected OR genotypes (PIs: 588296, 588484, 588530) and the genotype that was SU (PI: 588224). Prior to inoculation, berries were examined microscopically at 160x to ensure they were free of prior infection. The berries were placed pedicel-side down onto the rack of an empty pipette-tip box (Rainin Instrument, Oakland, CA) with a moistened filter paper in the bottom to maintain high humidity. Berries were inoculated by dusting them with heavily mildewed leaves with actively sporulating colonies and were incubated in the closed boxes at room temperature (25°C) for seven days. Berries were then examined using an epi-illuminescence microscope (Zeiss Model 14, Göttingen, Germany) at 160x magnification without further processing or staining. Three hundred fifty conidia per genotype were observed and categorized as 1) germinated with an appressorium, 2) with a non-branched secondary hypha, or 3) with branched secondary hyphae. Conidia with a non-branched secondary hypha were not included in data analysis since they could arise either from a successful penetration or by a conidium capable of growing a second germ tube after the first was unsuccessful. OR genotypes were grouped and compared to the SU genotype. A χ^2-test of difference between two proportions was used to determine if a significant difference existed between the proportion of conidia successfully infecting (assessed by the presence of branched secondary hyphae) and those unsuccessful, compared between the resistant and susceptible groups. All replicate experiments were combined and the ratio of successful and unsuccessful penetration events was compared between the OR group and the SU genotype.

Berry size measurement. In 2006, one uninoculated cluster was marked on each replicate vine for each of eight selected genotypes (PIs: 483176, 588224, 588225,

588231, 588258, 588296, 588484, 588530) to monitor berry growth. Prior to bloom, a portion of the cluster containing approximately 20 berries was partitioned using Teflon tape tied loosely around the rachis. Berry diameter was measured using digital calipers, taken at the widest part of the berry every 4 days starting at bloom. For each cluster, the two largest diameter values within the partition were averaged to accommodate for the presence of shot berries. The measurement date was converted to GDD at each measurement (see above).

RESULTS

Phenotypic screen and genotypic categorization. For berries, 50 genotypes were resistant at every time-point (CR), one genotype remained susceptible throughout the time points tested (SU), and 24 genotypes exhibited a significant gain of resistance (OR) (Figures 1.1, 1.2), and four genotypes exhibited no statistically significant pattern (NS) (Tables 1.1, 1.2). Categorization for the rachis portion of the cluster did not correspond with berry categorization - of the 79 genotypes, 13 genotypes were classified as CR, 41 as SU, seven as OR, and 18 as NS (Tables 1.1, 1.2). Similarly, the categorization of resistance in the pedicel portion of the cluster differed from other tissues with four genotypes being classified as CR, 41 as SU, 13 genotypes as OR, and 21 as NS (Tables 1.1, 1.2).

Figure 1.2. Clusters from *Vitis hybrid* 'NY 65.591.1' (PI: 588484) exhibiting the development of ontogenic resistance. Clusters were field inoculated, from left to right, at 8, 15 and 21 days post-bloom. The earliest inoculation date resulted in extensive disease, the middle date intermediate disease, and no disease on the latest. Clusters were collected following six or more weeks of incubation on the vine. Photo by Joe Ogrodnick.

Table 1.2. Genotypes classified as constitutively resistant

PI[a]	*Vitis* species[b]	Variety[c]	Average severity (%)[d]	Rachis Classification[e]	Pedicel Classification[f]
588399	*V. acerfolia*	150-44	0.4	SU	SU
588324	*V. acerifolia*	unknown	0	SU	SU
588325	*V. acerifolia*	unknown	0	CR	NS
588218	*V. cinerea*	B 47	1.3	SU	NS
588220	*V. cinerea*	B 55	1.2	SU	SU
588222	*V. cinerea*	C-66-7	0	SU	NS
483176	*V. hybrid*	Tom's Favorite	0.4	SU	OR
588563	*V. hybrid*	Minn 78	0	CR	OR
588572	*V. hybrid*	R 67-76	0	SU	SU
588573	*V. hybrid*	D-1-30	0.3	SU	SU
483145	*V. labrusca*	Rem 26-75	0	NS	NS
483148	*V. labrusca*	Rem 46-75	0.6	CR	NS
483150	*V. labrusca*	Rem NE 8	0.4	NS	NS
483152	*V. labrusca*	Rem NE 11	0	CR	NS
483155	*V. labrusca*	Rem NE 15	0	CR	OR
483158	*V. labrusca*	Rem NE 23	0	CR	CR
483160	*V. labrusca*	Rem NE 25	1	CR	OR
255189	*V. riparia*	Urbana 1	0	OR	OR
483165	*V. riparia*	Rem 55-75	0	CR	NS
483170	*V. riparia*	Rem 73-76	0	SU	SU
483171	*V. riparia*	Rem 77-76	0.3	NS	NS
483172	*V. riparia*	Rem 81-76	0	NS	SU
483173	*V. riparia*	Rem 82-76	0.3	SU	NS
483174	*V. riparia*	Rem 83-76	0.3	SU	SU
483175	*V. riparia*	Rem 85-76	0.7	CR	NS
588258	*V. riparia*	14	0.4	SU	SU
588259	*V. riparia*	37	0	SU	SU
588260	*V. riparia*	64	0	SU	SU
588261	*V. riparia*	74	0	SU	SU
588262	*V. riparia*	89	0	OR	SU
588269	*V. riparia*	62-8-160	0	NS	CR
588270	*V. riparia*	62-9-44	0	NS	NS
588274	*V. riparia*	62-11-42	1.3	NS	NS
588276	*V. riparia*	62-8-138	0	NS	NS
588304	*V. riparia*	unknown	0	SU	NS
588347	*V. riparia*	B 50	0	CR	OR
588353	*V. riparia*	RA-66-7	0.1	NS	NS
588400	*V. riparia*	unknown	0	CR	CR
588406	*V. riparia*	Meissner 13	0.4	NS	SU
588435	*V. riparia*	2F	0	NS	OR
588437	*V. riparia*	1F	0	OR	SU
588438	*V. riparia*	unknown	0.2	NS	NS
588455	*V. riparia*	unknown	0	SU	NS
588456	*V. riparia*	unknown	1.2	SU	SU

Table 1.2 (continued)

588483	*V. riparia*	Rem NE 21	0.1	CR	OR
588565	*V. riparia*	Grem	0	NS	SU
588568	*V. riparia*	Crosby rootstock	0	SU	SU
588392	*V. x andersonii*	unknown	0	SU	SU
588391	*V. x champinii*	unknown	0.6	OR	OR
588257	*V. x novae-angliae*	unknown	0	CR	CR

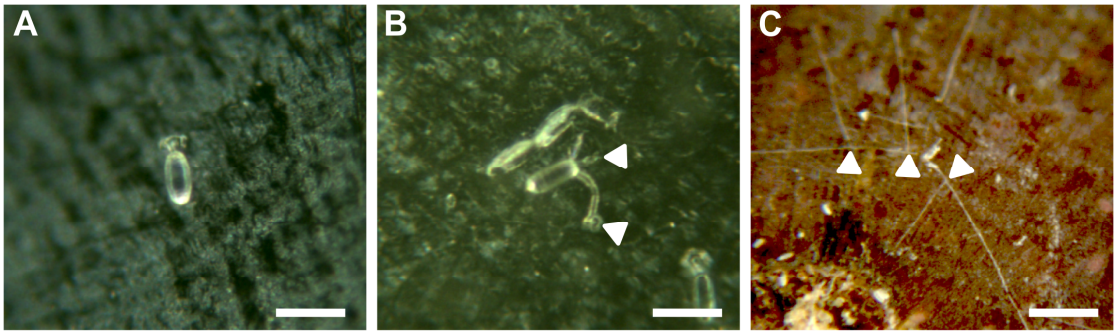
[a.] PI = Plant Introduction number, a unique identifier for each genotype in the GRIN database, where PI is linked with additional information related to that genotype: http://www.ars-grin.gov/npgs/index.html (13).
[b.] Species listed for each genotype, taken from the GRIN database. *Vitis* interspecific hybrids are listed as *V. hybrid*.
[c.] Variety listed for each genotype, taken from the GRIN database. Genotypes with no variety recorded listed as unknown.
[d.] Average disease severity of all clusters, average among all years
[e.] Classification of the rachis using the same criteria developed for berry data.
[f.] Classification of the pedicel using the same criteria developed for berry data.

For berries, the average maximum severity was much higher in the OR group (20.3%) than the CR group (0.5%). For normally-developing CR berries, 1104 of 1137 inoculated clusters had 0% disease severity across the three years. However, CR shot berries were frequently infected with powdery mildew (461 of 1137 clusters), and this susceptibility did not typically diminish with time as 40 of the 47 CR genotypes with susceptible shot berries retained this susceptibility through 28 DPB.

Berries of the single SU genotype exhibited the highest average maximum severity at 40.2%. By species, the four *V. rupestris* (17.7%) and the 26 genotypes of interspecific hybrids including *V.* x *andersonii*, *V.* x *champinii* and *V.* x *novae-angliae* exhibited the highest average maximum severity (16.8%) while the *V. riparia* (1.0%) and *V. acerfolia* (0.0%) exhibited the lowest maximum severity. For OR genotypes, resistance was gained in a development period ranging from 21.4 GDD to 284.3 GDD (Table 1.1). Only four of 230 uninoculated control clusters exhibited any disease, and the average overall severity of control clusters was 0.2%, reflecting very low background levels of powdery mildew in the vineyard.

Conidial germination and colony formation on putatively resistant berries. The proportion of branched secondary hyphae was significantly lower in the OR group than in the SU genotype ($P = 0.0005$). For the SU genotype *V. rupestris* 'R-65-44' (PI: 588224), 16.2% of germinated conidia exhibited relatively long and extensively branched secondary, tertiary, and higher-order hyphae (Figure 1.3C). On genotypes classified as OR, mildew colonies failed to develop from germinated conidia within seven days after inoculation (*e.g.* Figure 1.3A).

Figure 1.3. Epi-illuminescence micrographs showing the three categories of germinated conidia used to classify pathogen growth on detached berries. Individual germinated conidia were classified as developing as follows: (A) a germinated conidium with a primary appressorium but no further growth; (B) a germinated conidium with a primary appressorium and secondary hyphae (arrows); and (C) a germinated conidium showing extensive and branching secondary hyphae (arrows). Note the berry pigmentation in (C) reflecting the post-veraison status of this persistently susceptible, black-skinned genotype, *V. rupestris* 'R-65-44' (PI: 588224). Scale bars represent 50 μm in panels A and B, and 100 μm in panel C.


A
B
C

No conidia were able to infect *V. hybrid* 'NY 65.591.1' (PI: 588484) or *V. hybrid* 'NY 65.556.5' (PI: 588530). While some conidia (3.2%) overcame penetration resistance on berries of *V. hybrid* 'Red Amber' (PI: 588296), growth did not expand beyond 10 μm of the initial branch point.

Berry size measurements. Berries from *V. rupestris* 'R-65-44' (PI: 588224) expanded in a manner similar to all genotypes measured including other *V. rupestris* genotypes (PIs: 588225, 588231) (Figure 1.4). Further, *V. rupestris* 'R-65-44' shoots and clusters are characteristic of *V. rupestris*, regardless of phenotypic categorization (Figure 1.5).

DISCUSSION

Ontogenic resistance confers broad-spectrum and durable resistance to several grapevine diseases, including powdery mildew [4, 9-11, 13, 14, 21], downy mildew [14], and black rot [13]. Our goal was to identify variation for ontogenic resistance within a diverse collection of *Vitis spp*. Among 79 genotypes representing six *Vitis spp.* and several interspecific hybrids, for genotypes exhibiting any susceptibility, ontogenic resistance was highly conserved. However, one genotype (*V. rupestris* 'R-65-44') was found to have persistent and reproducible susceptibility (*i.e.* young berries were highly susceptible and did not acquire resistance as they aged).

Constitutive resistance. Of the genotypes screened in the current study, the majority (50 out of 79) were resistant at all points of development (Table 1.2). Additionally, disease severity on berries was 0% in 1104 of 1137 CR clusters rated, reflecting strong resistance in these genotypes.

Figure 1.4. Berry size development of persistently susceptible *Vitis rupestris* 'R-65-44' (PI: 588224) is similar to ontogenically resistant and constitutively resistant genotypes. Berry diameter was measured every four days from the same 20 berries on one cluster on each adjacent replicate vine, and the largest two berries from each measurement were averaged to eliminate the influence of non-developing (shot) berries on means. Data were collected for the genotypes: *V. hybrid* 'Tom's Favorite', PI: 483176; *V. riparia* '14', PI: 588258; *V. rupestris* 'R-65-44', PI: 588224; *V. rupestris* 'R-65-47', PI: 588225; *V. rupestris* 'Alphonse de Serres', PI: 588231; *V. hybrid* 'Red Amber', PI: 588296; *V. hybrid* 'NY 65.591.1', PI: 588484; and *V. hybrid* 'NY 65.556.5', PI: 588530. One replicate of *V. riparia* '14' (588258s) consisted entirely of shot berries. Error bars represent plus or minus one standard deviation.

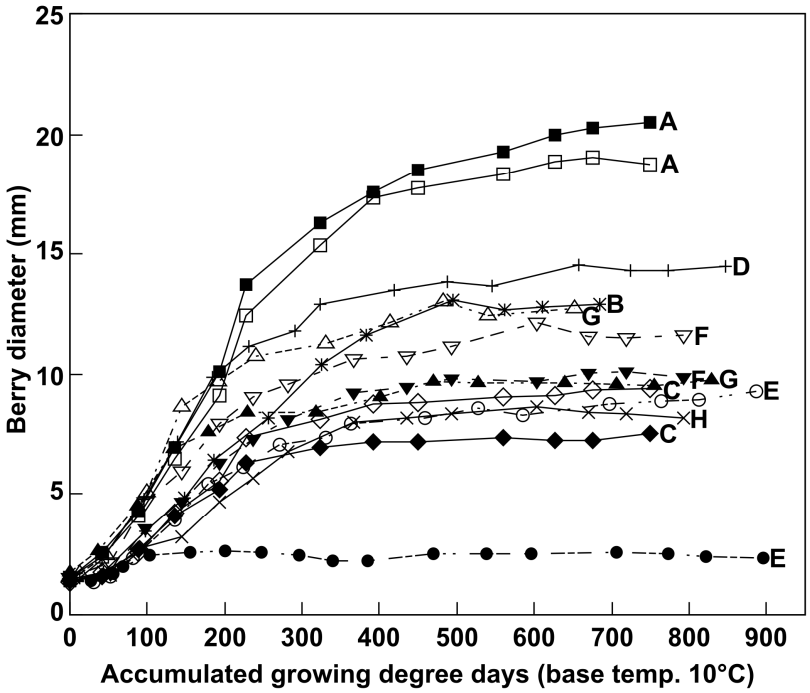
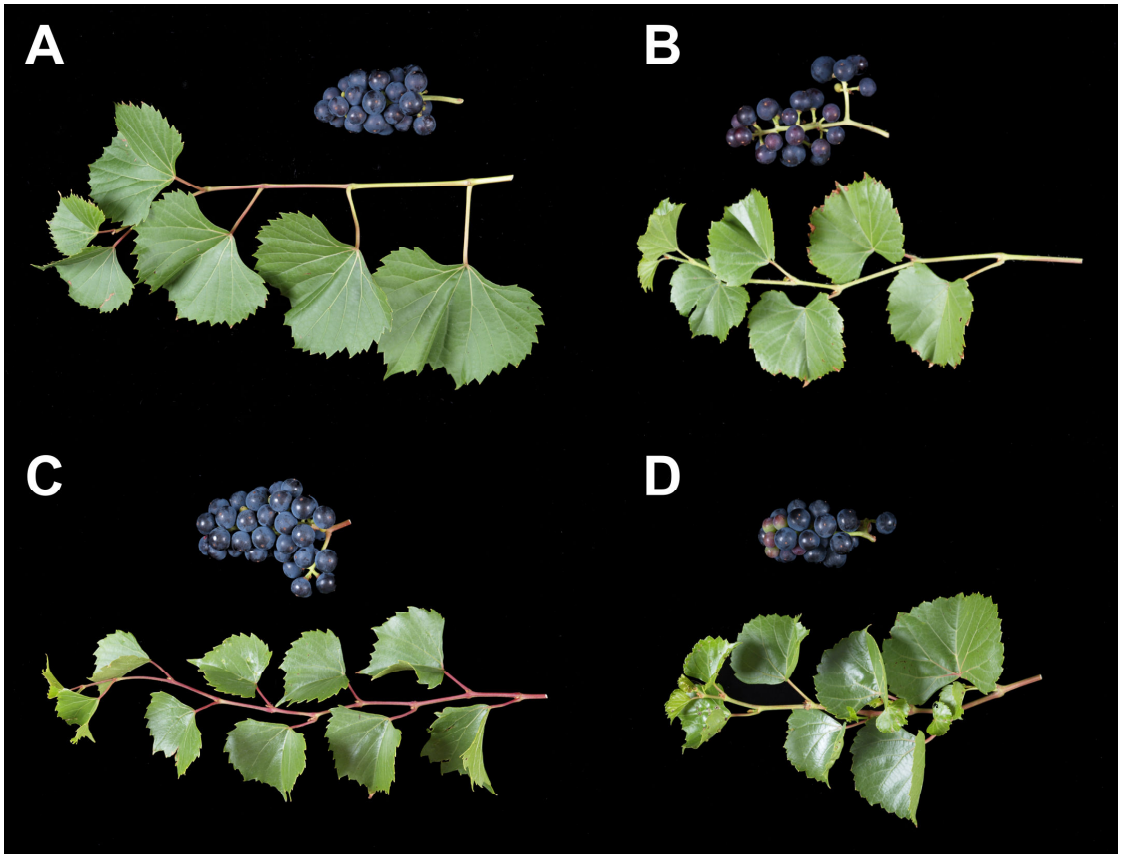

Berry diameter (mm)
Accumulated growing degree days (base temp. 10°C)
0
5
10
15
20
25
0
100
200
300
400
500
600
700
800
900
A
A
D
B
G
F
F
G
E
C
H
C
E

Figure 1.5. Clusters and shoots from the four genotypes of *V. rupestris* included in this study. The single SU genotype (A) *Vitis rupestris* 'R-65-44' (PI: 588224) undergoes normal development and is representative of the species *V. rupestris*. The other genotypes presented for comparison are (B) *V. rupestris* 'R-65-47' (PI: 588225); (C) *V. rupestris* 'Alphonse de Serres' (PI: 588231); and (D) *V. rupestris* 'RU-66-2' (PI: 588401). Photos by Joe Ogrodnick.


A
B
C
D

This frequency of resistance is not surprising since the germplasm evaluated was highly enriched for wild-genotypes of North American *Vitis* species (*e.g. V. riparia, V. cinerea, V. labrusca*), which are thought to have co-evolved with powdery mildew [17, 20]. Co-evolution has been previously demonstrated to enrich the frequency of natural resistance to pests [19].

For investigations of ontogenic resistance, CR genotypes do not provide insight, as there is little or no observable change in susceptibility. However, as a source of powdery mildew resistance for breeding programs, genotypes that exhibited CR could be useful, particularly the three genotypes (PIs: 483158, 588257, 588400) that were resistant in all tissue types of the cluster. Seven interspecific hybrids were found to be CR in the berries (Table 1.2), and none of these pistillate-flowered genotypes have *V. vinifera* documented in their pedigree [23].

Ontogenic resistance. On the other hand, of the genotypes classified as OR, the majority (17 out of 24) were interspecific hybrids (Table 1.1). The primary documented source of ontogenic resistance is the European grape (*V. vinifera*) [8, 11, 21], which is also thought to be a source of hermaphroditism in grapevine [22]. Fifteen of the 17 *V. hybrid* genotypes classified as OR have hermaphroditic flowers (Gee and Cadle-Davidson, unpublished), suggesting *V. vinifera* as the source of OR in these individuals. Ontogenic resistance requires susceptibility at some point in tissue development, and *V. vinifera* genotypes in general are excellent donors of powdery mildew susceptibility.

Ontogenic resistance of grape berries was not confined to *V. vinifera* and related genotypes, but was also identified in berries of individual genotypes of native North

American grapevines *V. labrusca*, *V. riparia*, and *V. rupestris*, suggesting broad conservation of the trait in susceptible individuals of European and some North American species (Table 1.1). These North American genotypes retained quantitative levels of resistance during their susceptible phase, such that the maximal severity of powdery mildew was lower in the North American OR genotypes than in the *V. hybrids* in the OR group (Table 1.1). As an exception to this, the highest severity occurred in the persistently susceptible *V. rupestris* 'R-65-44'. Thus, absence of ontogenic resistance in this genotype resulted not only in a protracted period of susceptibility, but the amplitude of susceptibility was elevated above all other genotypes.

In the search for genotypes with prolonged susceptibility, we did not expect to identify a phenotype as extreme as that for *V. rupestris* 'R-65-44', which maintains berry susceptibility past veraison on detached berries and much later than any other grapevine tested (Table 1.1). Further, persistent and heightened susceptibility was unexpected in *V. rupestris*, which is widely characterized as a powdery mildew-resistant *Vitis* species. Therefore, we checked vine and cluster morphology and confirmed that this genotype had apparently normally developing berries and exhibited other phenotypic traits exemplifying *V. rupestris*, such as red stems and leaf petioles, a bushy habit with shortened internodes, and imperfect flowers [23] (Figures 1.4, 1.5).

For *Vitis vinifera* 'Chardonnay' and *Vitis labruscana* 'Concord', ontogenic resistance has been shown to prevent *U. necator* from penetrating beyond the berry cuticle [9]. Our results suggests this mechanism is also present in OR individuals in this study. Ontogenically resistant berries significantly inhibited haustorium formation and

secondary hyphal growth relative to persistently susceptible *V. rupestris* 'R-65-44' (P=0.0005) (Figure 1.3). In one genotype, *V. hybrid* 'Red Amber', ontogenic resistance allowed a small proportion of conidia to penetrate, but colony establishment quickly failed. This observation reveals that ontogenic resistance may be expressed at different rates or to different degrees during infection.

Tissue specificity. In this diverse collection, we observed a large disparity in phenotypic classification between sections of the cluster (*i.e.* rachis, pedicel, berry). Although Concord was previously shown to develop OR in multiple tissue types but with tissue-specific timing [10], the diverse genotypes in the current study rarely had the same response in all tissues, with only four of 79 genotypes having the same classification on all three tissue types (Tables 1.1, 1.2). In part, this could be explained by the frequent occurrence of susceptibility exhibited in the rachis and pedicel portions of clusters in this collection. While only one genotype had persistently susceptible fruit, 50 genotypes had persistently susceptible rachises and/or pedicels. This finding opens new avenues of research into the mechanism of ontogenic resistance. Having genotypes that express susceptibility asynchronously among the various tissue-types of the cluster could allow for reduction of environmental effects, compared to studying clusters from different vines or even from the same vine. It should be noted, however, that our method for describing the resistance phenotypes of berries was not as successful in categorizing the rachis and pedicel phenotypes. While the berries of only four genotypes were classified NS, 21 and 18 genotypes were NS for the pedicel and rachis respectively. One aspect that caused several genotypes to have NS pedicel and rachis phenotypes was an apparent gain of susceptibility (data not shown).

Shot berries. Many genotypes in this study exhibited high proportions of undeveloped berries (*i.e.,* "shot berries") that arose from failed fertilization events, were stunted in growth and development, and typically remained small and green all season [5]. Interestingly, these berries remained susceptible through the growing season, and, surprisingly, shot berries on many CR genotypes remained fully susceptible, regardless of the inoculation time. Additionally, even during the earliest stages of berry development (0-6 DPB) on CR genotypes, there were differences between shot berries and normally developing berries that allowed infection and colonization of unfertilized flowers. Thus, aspects of fertilization or seed development may be somehow linked to resistance, and this may provide an additional avenue by which the genetic basis of ontogenic resistance could be investigated. Due to shot berries having susceptibility unrepresentative of normally-developing berries, we did not include data from shot berries in our analyses. A potential benefit of selecting against shot berry production in breeding programs might be to enhance the impact of ontogenic resistance by eliminating such persistent inoculum sources.

This project disclosed a substantial amount of temporal and tissue-specific variation in susceptibility to powdery mildew. For investigating the basis of ontogenic resistance, some of this variation could prove useful. By identifying genotypes that are either OR (including several that appear to be temporally distinct) or SU, the basis of ontogenic resistance could be uncoupled from other aspects of berry development occurring synchronously. Further, the discovery of a persistently susceptible genotype will allow for genetic studies to be carried out investigating the inheritance of ontogenic resistance.

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CHAPTER 2

Proteomic analysis of early grape berry development using *in silico* phenotypic bulking to investigate protein expression related to ontogenic resistance

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ABSTRACT

Understanding the basis of ontogenic resistance in grape berries would potentially facilitate further exploitation of this durable form of disease resistance. Technologies for studying protein expression provide one method to increase our understanding of this phenomenon. We used gel-based proteomics with *in silico* phenotypic bulking of protein samples from non-related genotypes of *Vitis spp.* to identify proteins whose expression was correlated with development of ontogenic resistance. To correlate protein expression with development of ontogenic resistance, uncoupled from normal berry development, we compared three genotypes of *Vitis* interspecific hybrids that exhibited the typical developmental pattern for ontogenic resistance and a single genotype whose berries remained susceptible. One hundred forty-two proteins were successfully identified, including 55 hypothetical proteins in the *Vitis vinifera* genome, and were either specifically correlated to the gain of ontogenic resistance, conserved in expression during berry development, or developmentally regulated in a genotype-dependent manner. The majority of these proteins were involved in metabolic and photosynthetic pathways, or in gene-regulation. Of specific interest, proteasome activity, previously shown to affect powdery mildew resistance, was up-regulated through development in the OR genotypes. This and other identified candidate proteins provide the opportunity to enhance and broaden the impact of ontogenic resistance in new, improved cultivars, while adding further value to grapevine genomic resources by identifying proteins correlated with early berry development.

INTRODUCTION

Grapevine (*Vitis spp.*) is one of the most widely planted fruit crops accounting for 7.4 million hectares worldwide producing 68 million metric tons of harvested fruit annually [27]. Grapes are also high-value, with wine sales alone contributing over $160 billion to the economy of the United States each year [44]. Its status as a high-value crop combined with the fact that most of the important varieties (*e.g. V. vinifera* cultivars, *V. labruscana* 'Concord') have high levels of susceptibility to prevalent and highly-destructive pathogens [12, 20, 26] make research into new avenues of improving host resistance important.

One type of resistance that has been the focus of several studies is ontogenic resistance (OR), which is typified by a period of susceptibility beginning at anthesis and rapidly decreasing over 2-3 weeks until a near-immunity is achieved [15]. This resistance has been found to be effective toward several important pathogens [10, 13-15, 37].

Previous investigations into the underlying mechanism of OR showed that several factors common in plant-host defense were not involved in OR, including: cuticle and anticlinal cell wall thickness, papillae formation, expression of PR-1 or a germin-like protein, accumulation of polyphenolics, or factors controlling conidial spore attachment or germination [9, 11].

Although all cultivated grapes develop OR similarly, a single genotype (*V. rupestris* 'R65-44') has been identified that remains persistently susceptible to powdery mildew throughout early berry development and beyond the onset of ripening at veraison [15]. This genotypic variation for the presence of OR provides an unprecedented

opportunity to identify the genetic basis, but populations segregating for OR are not yet available.

The first month of grape berry development is a physiologically active period of rapid cell division, cell expansion, and photosynthesis. While no studies have looked at protein expression in the first month of grape development, proteomic analyses have been published related to ripening in this important non-climacteric fruit system [5, 16]. Proteomic analysis of berry peels has provided insight into development of the complex chemical nature desired in wine and table grapes [3, 7]. Further, berry peels are relevant to the development of peripheral berry structures such as the cuticle and anticlinal cell wall, which is the interaction site between grape and powdery mildew, thus making them relevant to the development of OR [9, 11]. Using berry peels in proteomics also aids in increasing resolution of protein expression determination through tissue-specific fractionation [8, 40]. Proteomic studies can be hindered by limited availability of gene sequence data; however, the recent release of the *V. vinifera* [19] genome provides a valuable tool for conducting these types of studies.

The goal of the current study was to uncouple OR-related protein expression from the non-resistance-related expression occurring in the dynamic first month of grape berry development. This uncoupling was carried out using a method of *in silico* bulking of protein gel images based on resistance phenotype, an approach previously used to compare insect resistance in a genetically diverse population of rice [31]. The current study used four genetically distinct genotypes of grapevine, three that developed OR and the persistently susceptible genotype R65-44 [15].

METHODS AND MATERIALS

Vines and tissue handling. Four genotypes of 20 year-old field-grown vines at the USDA-ARS cold-hardy *Vitis* germplasm collection located in Geneva, New York were used for these experiments. These included three genotypes that exhibited a typical OR: *V. hybrid* 'Red Amber' (PI: 588296), *Vitis hybrid* 'NY 65.591.1' (PI: 588484), and *V. hybrid* 'NY 65.556.5' (PI: 588530), and one genotype *V. rupestris* 'R-65-44' (PI: 588224), which remained susceptible (SU) throughout the growing season [15]. Plant introduction (PI) numbers correspond to the individual genotype records found in the USDA-ARS Germplasm Resources Information Network (GRIN) database [41]. Each genotype was represented by two adjacent, replicate, own-rooted vines planted on 1.8 m centers using a three-wire trellis, cane-pruned and trained to the Umbrella Kniffin system. Clusters were harvested at predetermined time-points (Table 2.1) and submerged directly into liquid nitrogen for transport. Tissue was stored at -80°C until protein extraction.

Protein extraction and quantification. Grape berry skin peels were prepared for protein extraction by separating berries from the rachis, pedicels, and non-developing shot berries. Berry skin peels were then collected by allowing the berries to thaw slightly and removing the berry pulp before the tissue had thawed completely. For protein extraction, berry peels were placed in liquid nitrogen immediately after removing the pulp and ground to a fine powder using a mortar and pestle. Peels from a single cluster were weighed and split between different tubes for use as technical extraction replicates when the total mass was in excess of 2.5 g. All samples were handled identically for extraction and protein expression analysis.

Table 2.1. Samples used for proteomic study

Sample number	PI number[a]	Genotype[b]	Days Post bloom	Phenotype[c]
1	588296	*V. hybrid* 'Red Amber'	10	S
2	588296	*V. hybrid* 'Red Amber'	26	R
3	588296	*V. hybrid* 'Red Amber'	26	R
4	588296	*V. hybrid* 'Red Amber'	7	S
5	588296	*V. hybrid* 'Red Amber'	10	S
6	588296	*V. hybrid* 'Red Amber'	15	R
7	588224	*V. rupestris* 'R-65-44'	28	S
8	588224	*V. rupestris* 'R-65-44'	9	S
9	588224	*V. rupestris* 'R-65-44'	5	S
10	588224	*V. rupestris* 'R-65-44'	22	S
11	588224	*V. rupestris* 'R-65-44'	22	S
12	588224	*V. rupestris* 'R-65-44'	10	S
13	588224	*V. rupestris* 'R-65-44'	9	S
14	588530	*V. hybrid* ' NY 65.556.5'	28	R
15	588530	*V. hybrid* ' NY 65.556.5'	28	R
16	588530	*V. hybrid* ' NY 65.556.5'	28	R
17	588530	*V. hybrid* ' NY 65.556.5'	11	S
18	588530	*V. hybrid* ' NY 65.556.5'	11	S
19	588530	*V. hybrid* ' NY 65.556.5'	11	S
20	588484	*V. hybrid* ' NY 65.591.1'	9	S
21	588484	*V. hybrid* ' NY 65.591.1'	9	S
22	588484	*V. hybrid* ' NY 65.591.1'	21	R
23	588484	*V. hybrid* ' NY 65.591.1'	21	R
24	588484	*V. hybrid* ' NY 65.591.1'	21	R
25	588484	*V. hybrid* ' NY 65.591.1'	21	R
26	588484	*V. hybrid* ' NY 65.591.1'	10	S

Table 2.1 (continued)

[a.] PI = Plant Introduction number, a unique identifier for each genotype in the GRIN database, where PI is linked with additional information related to that genotype: http://www.ars-grin.gov/npgs/index.html [41].

[b.] Genotype name taken from the GRIN database. *V. hybrid* used to denote a *Vitis* interspecific hybrid.

[c.] Phenotype listed as (R) Resistant; (S) Susceptible. Phenotypes determined at time of tissue collection, taken from Gee et al. [15].

Total soluble protein was extracted using a technique modified from Hurkman and Tanaka [18]. Ground tissue was transferred to 15 mL conical bottom tubes (BD Falcon, San Jose, CA) containing 5 mL of extraction buffer (0.7 M Sucrose, 0.5 M Tris pH 7.5, 50 mM EDTA pH 8.0, 0.1 M KCl, 2% v/v β-mercaptoethanol, 1% w/v PVP40, 50 μg/ml Pefabloc SC (Sigma, St. Louis, MO)) and vortex-agitated to fully suspend the tissue into the buffer. To each tube, 2.5 mL of Tris-saturated phenol, pH 8.0 (Sigma, St. Louis, MO) was added, followed by vortex agitation until contents mixed. The tubes were shaken at moderate speed for 20 min on an orbital shaker at room temperature (RT), followed by phase separation under centrifugation (1568xg) for 30 min at 4°C. The phenolic (top) phase was removed to a separate 15 mL conical bottom tube and set aside. Extraction from the aqueous phase was repeated by adding an additional 2.5 mL of phenol, followed by agitation, shaking, and phase separation, as described above. The phenol layers from both extractions were combined, and contaminants were removed by two back-extractions, each entailing the addition of 5 mL extraction buffer, agitation, shaking, and phase separation, as described above. The resulting phenol layer was decanted to a clean tube and precipitated overnight (ON) with five volumes of 0.1 M ammonium acetate in methanol at -20° C. Proteins were recovered by centrifugation (4000xg) for 60 min at 4° C. The ammonium acetate was decanted, and the protein pellet was washed with ice-cold methanol twice and 80% ice-cold acetone twice, with a centrifugation (4000xg at 4° C) for 30 min after each wash, followed by drying under N_2-flux. Pellets were transferred using a spatula to 2 mL microfuge tubes and stored at -80° C until further use.

Pellets were solubilized in a modified R2D2 buffer (R2D2') [24], with the dithiothreitol (DTT) concentration increased to 50 mM and the Tris(2-carboxyethyl) phosphine (TECP) replaced with 2 mM tributyl phosphine (TBP) [17]. Upon addition

of R2D2', samples were agitated by vortexing at RT to aid solubilization of the pellet. Protein samples were quantified using the RC-DC Protein Assay kit (Bio-Rad, Hercules, CA) using the manufacturer provided microplate protocol, modified with an addition precipitation and solubilization cycle to ensure good contamination removal. The solution was transferred to a 96-well microplate for reading absorbance using a plate reader (BioTek, Winooski, VT) at 750 nm. Bovine Serum Albumin (Bio-Rad, Hercules, CA) was used to create a standard curve of 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 μg to use for concentration determination. The standard curve was prepared at the same time with the samples to be quantified.

Sample labeling and 2D-SDS-PAGE. For Differential in-Gel Electrophoresis (DIGE) (GE Healthcare, Piscataway, NJ) analysis, all samples were randomly paired for electrophoresis (Table 2.2). Samples were labeled individually with Cy3 or Cy5 Cy Dye fluor (GE Healthcare, Piscataway, NJ) and run in duplicate, totaling four replicate images for each sample. Each DIGE gel contained a pooled, internal standard comprising equal masses of proteins from each sample in the experiment, labeled with Cy2. The pH of the sample cocktails was corrected for compatibility to 8.5 using unbuffered Tris (pH=10.8) applied prior to addition of Cy Dye. For each sample, 50 μg of proteins were labeled using a minimal-labeled strategy (Ettan DIGE System Manual, GE Healthcare, Piscataway, NJ) with 200 pmol of Cy Dye for 30 min on ice in the dark, followed by a 10 min quench with 10 mM lysine on ice in the dark. Cy2-, Cy3- and Cy5-labeled samples were combined with 2% of pH 3-10 carrier ampholytes and 20 mM DTT (GE Healthcare, Piscataway, NJ). Sample cocktails were brought to a final volume of 450 μl with an isoelectric focusing (IEF) solution containing 7 M urea, 2 M thiourea, and 4% CHAPS [30].

Table 2.2 Gel sample combinations		
Gel number	Cy3 sample	Cy5 sample
1	8	4
2	4	13
3	12	1
4	13	5
5	5	12
6	1	8
7	5	8
8	7	1
9	7	2
10	1	11
11	12	10
12	9	12
13	8	4
14	3	3
15	6	3
16	10	9
17	6	5
18	10	10
19	3	6
20	4	11
21	11	9
22	9	7
23	11	6
24	13	2
25	2	7
26	2	13
27	26	21
28	20	26
29	22	20
30	23	21
31	21	20
32	22	23
33	20	26
34	23	22
35	20	23
36	26	20
37	11	9
38	24	20
39	21	11
40	24	15
41	11	25
42	18	19
46	14	18
47	14	17
48	17	16

The IEF solution was incubated prior to cocktail construction with AG 501-X8(D) mixed-bed ion-exchange resin (Bio-Rad, Hercules, CA) to remove ammonium cyanate and minimize the possibility of co-analytical modification of the proteins. Sample cocktails were centrifuged (15,000xg at 25° C) for 10 min to remove non-dissolved particles. Bromophenol blue was used as the tracking dye during all steps of electrophoresis. Samples were applied to pH 4-7, 24 cm Immobiline DryStrips (GE Healthcare, Piscataway, NJ) using passive diffusion ON at RT using the Immobiline DryStrip Reswelling Tray (GE Healthcare, Piscataway, NJ).

Isoelectric focusing was carried out with the Ettan IPGphor II (GE Healthcare, Piscataway, NJ), using the following parameters: 500 V for one hour, ramp to 1000 V for six hours, ramp to 8000 V for three hours, and hold at 8000 V until a total of 100,000 Vh was reached. Following IEF, strips were either prepared immediately for 2^{nd} dimension electrophoresis or stored at -20° C until further use.

Prior to 2^{nd} dimension electrophoresis, proteins were reduced and then alkylated using a buffer containing 50mM Tris pH 8.8, 6M Urea, 30% (v/v) Glycerol, and 4% SDS with 2% DTT for reduction and 2.5% iodoacetamide for alkylation, Bromophenol blue was used as a tracking dye. Strips were incubated in 24 cm strip tubes (GE Healthcare, Piscataway, NJ) with slow shaking for 15 min each round at RT.

Proteins were resolved in either 24 x 20 x 0.1 cm, 12.5% poly-acrylamide gels cast using the DALT*six* gel caster (GE Healthcare, Piscataway, NJ), or pre-cast 24 x 20 x 0.1 cm, 12 % acrylamide gels (Jule Inc., Milford, CT) using the Ettan DALT*six* vertical gel apparatus and EPS 601 power supply (GE Healthcare, Piscataway, NJ). Following reduction and alkylation, strips were placed on the top edge of the gel and

sealed with 1% agarose. Gels were run at 5 W per gel for 30 min, followed by 16 W per gel until the dye-front was 5 mm from the bottom of the gel. The apparatus was cooled using a circulating waterbath set to 25° C. Electrophoresis buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS) was used for the anode chamber. SDS concentration was increased to 0.2% in the cathode chamber as per the manufactures protocols (GE Healthcare, Piscataway, NJ).

Picking gels were run under the same conditions as the DIGE analysis gels, except using 700 μg of the internal standard cocktail, which was not labeled with Cy Dye. Gels were 24 x 20 x 0.1 cm 12% precast gels, with bind silane on the tall plate, and repel on the short plate (Jule Inc., Milford, CT). Following electrophoresis, picking gels were stained using Deep Purple stain following the manufacturer-supplied improved protocol (GE Healthcare, Piscataway, NJ).

Gel imaging and software analysis. DIGE gels were imaged with a Typhoon 9400 Variable Mode Imager (GE Healthcare, Piscataway, NJ) in fluorescence acquisition mode with a 100 micron scan resolution, adjusting the scan voltage to prevent image saturation. All DIGE images were analyzed using Progenesis Samespots v.2.0 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK), which was used for gel alignment, grouping of gels, and statistical (e.g. ANOVA and power analysis) and spot expression analysis. Gel images were initially organized into groups based on the phenotypic state (*e.g.* resistant or susceptible) of the tissue at time of harvest (phenotypic bulks). This setup resulted in the 'susceptible bulk' containing gels with proteins from young and old tissue of the SU genotype and young tissue of the OR genotypes while the 'resistant bulk' only contained sample representatives from old tissue of the OR genotypes.

Ninety-three spots were selected for excision from a picking gel and trypsin digestion based on having at least ±1.5-fold change of expression between the phenotypic bulks and a significant ANOVA statistic (≤ 0.05). Spot volumes normalized to the internal Cy2 control were used for all fold-change determinations. Prior to protein identification with mass spectrometry, individual spots were analyzed separately for quantitative expression changes during the development of each genotype. Spots were selected for mass spectrometry based on available resources, with care to include all spots belonging to groups 1 and 2, defined as follows.

Spots were categorized into six expression groups, defined as follows: (1) spot volume higher in at least 2 of 3 OR genotypes compared to the SU genotype expression; (2) spot volume higher in SU genotype than in at least 2 of 3 OR genotypes; (3) spot volume does not significantly change during development in at least 3 of 4 genotypes, regardless of phenotypic class; (4) spot volume increases during berry development in at least 3 of 4 genotypes, regardless of phenotypic class; (5) spot volume decreases during berry development in at least 3 of 4 genotypes, regardless of phenotypic class; (6) spot not fitting into classes 1-5.

Spots picking and in-gel digestion for picked 2D gel spots. Gel spots were excised from picking gels using a ProPic robot (Genomic Solutions, Ann Arbor, MI). Progenesis Samespots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) was used to make triangulations between the reference gel in the primary analysis (based on phenotypic bulking) and the picking gel for generation of the necessary spot-coordinate file.

Picked protein spots in a 96-well plate were digested with modified trypsin (Promega,

Madison, WI) and extracted into a 96-well collection plate using the ProPrep liquid handling robot (Genomic Solutions, Ann Arbor, MI) for subsequent MS analysis following a protocol modified from Shevchenko, *et al*. [36]. All gel-extracted supernatants in the plate were evaporated to dryness in a Speedvac SC110 (Thermo Savant, Milford, MA).

Protein identification by nanoLC/MS/MS analyses. The 2-D spot samples were reconstituted in 10 μL of 2% acetonitrile (ACN) with 0.1% formic acid (FA) for LC-ESI-MS/MS analysis. NanoLC was carried out by an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). The gel extracted, digested peptides (6.4 μL) were injected using a Famous autosampler onto a C18 PepMap trap column (5 μm, 300 μm × 5 mm, Dionex) for on-line desalting and then separated on a PepMap C-18 RP nano column, eluted in a 30-minute gradient of 5% to 45% ACN in 0.1% formic acid at 250 nL/min The nanoLC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap from ABI/MDS Sciex (Framingham, MA) equipped with Micro Ion Spray Head ion source.

MS data acquisition was performed using Analyst 1.4.1 software (Applied Biosystems, Foster City, CA) in the positive ion mode for information dependant acquisition (IDA) analysis. The nanospray voltage was 2.0 kV used for all experiments in positive ion mode. Nitrogen was used as both the curtain (value of 10) and collision gas (set to high), with the heated interface on. The declustering potential was set at 50 eV, and Gas1 was 15 (arbitrary unit).

In IDA analysis, after each survey scan for *m/z* 400 to *m/z* 1550 and an enhanced

resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied for detected ions based on different charge states and *m/z* values. The MS/MS data generated from LC/ESI-based IDA analysis were submitted to Mascot 2.2 for database searching using in-house licensed Mascot local server, and the search was performed to query to NCBInr (taxonomy: Viridiplantae) database and to its decoy database with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 1.5 Da and MS/MS tolerance was set to 0.6 Da. Carbamidomethyl modification of cysteine and a methionine oxidation were set as variable modifications. Protein summaries were filtered using MudPIT scoring and a significance threshold of $P<0.01$. Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM) provides significance scores based on the Mowse algorithm [29], with scores reflecting similarity and identity. Only those peptides with significance scores greater than "identity" were considered for the protein identifications. The top scoring match was reported when redundant records matched.

NanoLC-MALDI analysis. Samples containing peptides from the 2D gel spots were separated on an UltiMate chromatography system equipped with a Switchos, FAMOS autosampler, and Probot spotting robot (Dionex, Sunnyvale, CA). The gel-extracted peptides (6 μL) were injected onto a PepMap C18 trap column (5 μm, 300 μm × 5 mm, Dionex) and then separated on a PepMap C-18 RP nano column (3 μm, 75 μm × 15 cm), eluted using a 60-min gradient of 5% to 40% ACN in 0.1% TFA at 250 nL/min. After the chromatographic column, the flow was directed to the Probot spotting robot, where fractions were collected every 20 s on a 576-position OptiTOF sample plate and simultaneously mixed with 0.34 μL of matrix (7 mg/mL CHCA containing 25 fm [Glu1] Fibrinopeptide B—Glufib—as internal standard for mass

calibration). These samples were then subjected to MALDI MS/MS analysis using a 4700 Proteomics Analyzer equipped with TOF-TOF ion optics (Applied Biosystems, Framingham, MA) and 4000 Explorer version 3.6. The instrument was operated in 1 kV positive ion reflector mode and calibrated with Glufib (Applied Biosystems, Framingham, MA) as an internal calibrant. The laser power was set to 4500 for MS and 5200 for MS/MS with CID off. MS spectra were acquired across the mass range of 850–4000 Da with a minimum S/N filter of 25 for precursor ion selection. MS/MS spectra were acquired for the 15 most abundant precursor ions, with a total accumulation of 2000 laser shots.

All MS/MS spectra generated from nanoLC-MALDI were submitted for database searching using GPS Explorer Software using the Mascot search engine (v 2.2) to search the NCBI nr database (taxonomy: Viridiplantae). The search parameters used specified trypsin cleavage allowing for a single miscleavage and variable modifications of methionine oxidation and cysteine carboxyamidomethylation. The mass tolerance was 75 ppm. Each protein identified was required to have at least one unique peptide identification not shared with any other protein. We report only those proteins with a protein identification confidence interval of ≥95%.

Oxidation analysis to confirm residue identity. Confirmation of peptide identification in which there was an ambiguity introduced due to the possibility of the nearly isobaric amino acid residues F and $M^{\rightarrow o}$, the method of Yang and Thannhauser (2007) [45]. In this method the putative peptide identifications are tested by carrying out an on-slide chemical modification, such as peracid oxidation. If the proposed sequence contains an oxidizable residue and the predicted mass shift is observed after

treatment, the ambigious amino acid is a $M^{\rightarrow o}$, and if the predicted mass shift is not observed, the amino acid is an F.

Annotation of hypothetical proteins from *Vitis vinifera*. Identifications involving hypothetical proteins from *V. vinifera* were annotated for function by comparison to redundant protein matches from other organisms in the MASCOT searches. These matches were confirmed using the hypothetical protein sequence obtained from GenBank to search for identity using BLASTP (database: NCBInr) [1].

RESULTS AND DISCUSSION

Protein extraction and protein population. Using DIGE technology required modifications to the protein extraction protocols chosen for this study. With the use of TECP in the R2D2 buffer [24] and the requirement for a pH range 8-9 for successful covalent labeling of proteins with Cy Dye (DIGE user manual, GE Healthcare), a large quantity of Tris was needed to increase the pH from the initial 3.5. The high concentration of Tris and the trianionic nature of TCEP resulted in a sample salt concentration too high for IEF. Switching to TBP alleviated these issues, as well provided other benefits when used as a reductant for IEF [17].

Similarly, the high levels of reductants and detergents required to solubilize the proteins from grape berry peels produced chemical interference during protein quantification. Accuracy and consistency in quantification of the samples was critical due to the precision in protein loading required for accurate comparisons using DIGE. As a result, an additional precipitation-solubilization step was required to ensure adequate removal of these compounds before spectrophotometry.

Following these modifications, a large population of soluble proteins was extracted from developing berry peels (Figure 2.1). There were 977 spots resolved on the 2D DIGE gels, as determined by Progenesis Samespots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) (Figure 2.1). Even utilizing stringent extraction and preparation techniques (*e.g.* phenol protein extraction, R2D2' solution, high-speed centrifugation, treatment of solutions with ion-exchange resin), issues with protein spot resolution, especially in the pH 4-5 range, was observed (Figure 2.1). This situation has also been observed in other grapevine proteomics studies [2, 5, 34, 35, 42] and likely results from the complex chemical nature of grapevine [25].

Analysis of protein expression. Samples were analyzed using Progenesis Samespots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) with samples bulked *in silico* based on their phenotype status, either susceptible or resistant, regardless of genotype and developmental stage. The Samespots analysis identified 147 differentially expressed spots distributed across the pH and molecular weight range of the gel (Figure 2.1). Ninety-three prominent and accessible spots were excised from the picking gel, 61 of which were subsequently chosen for digestion and identification based on their expression pattern in the normalized phenotypic bulks and individual genotypes (Table 2.3). Sixty out of 61 spots had a power analysis value greater than 0.8, the threshold selected as acceptable. Spot 7 was the single spot not meeting this threshold, with a power value of 0.57. The power analysis describes the ability to detect differences, given those differences actually exist. The high power achieved in this study means enough sample replication existed to find the vast majority of differentially expressed spots. Of spots indentified, those from categories one and two were the most implicated in the gain of resistance, as these have differential expression patterns between the susceptible and ontogenic resistance genotypes.

Figure 2.1. A Cy2 standard reference gel showing spots selected for identification. Spots selected for digestion and identification using mass spectrometry are numbered according to Table 2.3. Spots were picked from a gel containing 750 μg of the internal standard pool resolved on a 12% 2D-PAGE stained with fluorescent stain (see methods). Spots containing the same identified protein are marked with matching symbols. The molecular weight markers represent the band locations of the BioRad Broad Range Ladder (BioRad, Hercules, CA).

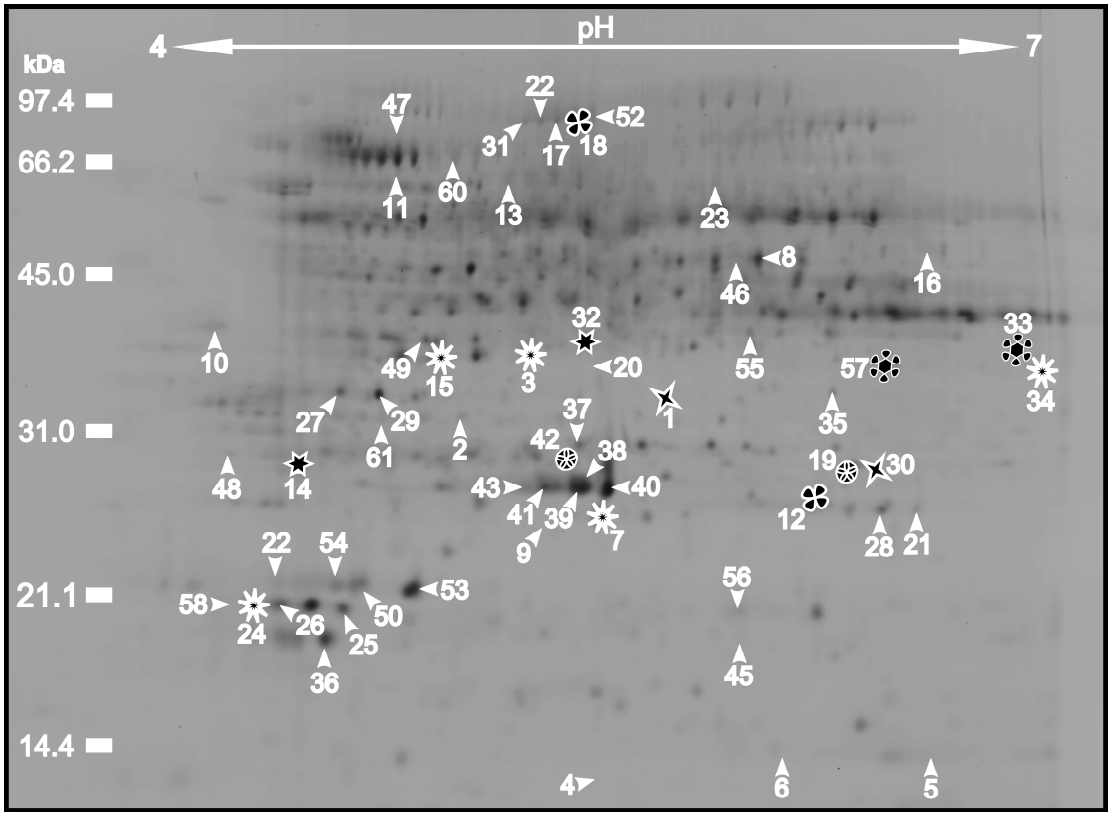
pH
4
7
kDa
97.4
66.2
45.0
31.0
21.1
14.4
47
22
52
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Table 2.3. Protein expression fold-change values for all Progenesis Samespots analysis

Spot number	Normalized spot volume fold-change relative to baseline samples[a]					Expression Group[b]	ID Obtained[c]	ID Method[d]
	Resistant Bulk	588224 Susceptible	588296 Resistant	588484 Resistant	588530 Resistant			
1	2.4	-1.1	2.5	1.7	1.5	1	yes	ESI
2	1.6	1.1	1.8	1.6	1.1	1	yes	ESI
3	-3.6	1.8	-1.0	1.1	-1.2	2	yes	ESI
4	-1.9	1.1	-1.6	1.0	-2.1	2	no	
5	-1.8	-1.2	-2.2	-1.6	-2.2	2	no	
6	-1.8	-1.2	-1.8	-1.2	-2.0	2	yes	LC
7	1.7	1.9	1.3	3.0	-1.3	2	yes	ESI
8	-1.6	-1.4	-1.8	-1.5	-1.3	2	no	
9	-1.6	1.2	-2.9	-1.0	-1.5	2	no	
10	-1.6	-1.3	-1.5	-1.2	-1.7	2	no	
11	-1.6	2.8	-1.7	-1.5	-1.2	2	yes	ESI
12	-1.6	1.3	-1.8	-1.6	1.6	2	yes	ESI
13	-1.6	-1.4	-1.9	-1.7	-1.0	2	yes	LC
14	-1.6	1.1	-1.9	-1.6	-1.3	2	yes	LC
15	1.5	1.6	1.3	1.4	-1.4	2	yes	ESI
16	-2.0	-1.3	-1.3	-1.3	-1.2	3	no	
17	-1.9	-1.1	-2.0	-1.3	-1.2	3	yes	ESI
18	-1.9	1.3	-1.7	-1.4	1.0	3	yes	ESI
19	-1.9	-1.1	1.1	-1.1	-1.2	3	yes	ESI
20	-1.8	1.5	1.2	-1.1	-1.3	3	yes	ESI
21	-1.7	1.3	-1.6	1.1	-1.1	3	yes	ESI
22	-1.7	-1.1	-1.8	-1.3	-1.0	3	yes	ESI
23	-1.7	-1.5	-1.4	-2.2	1.1	3	no	
24	1.7	1.3	-1.1	2.0	1.2	3	yes	ESI

Table 2.3 (continued)

25	-1.6	1.5	1.3	1.2	-1.2	3	yes	ESI
26	1.6	1.4	-1.1	1.5	1.1	3	yes	ESI
27	1.6	1.2	1.7	1.1	-1.4	3	no	
28	1.6	-1.3	1.4	1.6	-1.2	3	no	
29	1.6	1.4	1.8	1.4	1.2	3	yes	LC
30	-1.5	-1.0	-1.6	-1.1	-1.3	3	yes	ESI
31	-1.5	-1.2	-1.6	-1.3	-1.0	3	yes	LC
32	-1.5	1.6	-1.3	1.3	-1.3	3	yes	ESI
33	-1.5	-1.1	-2.2	-1.3	-1.2	3	yes	ESI
34	-1.5	1.4	-1.1	-1.3	-1.5	3	yes	ESI
35	3.0	2.0	4.1	4.5	1.4	4	no	
36	2.3	1.5	3.0	1.5	-1.3	4	yes	ESI
37	2.0	4.5	4.1	1.6	1.9	4	yes	ESI
38	2.0	4.1	4.5	2.1	1.8	4	yes	ESI
39	1.9	2.9	2.6	1.4	1.6	4	yes	ESI
40	1.8	3.2	3.3	6.2	1.9	4	yes	ESI
41	1.8	1.9	2.1	1.5	1.4	4	yes	ESI
42	1.8	2.7	2.7	1.0	1.5	4	yes	ESI
43	1.6	1.8	1.5	1.5	1.5	4	no	
44	1.6	1.9	2.1	1.7	1.4	4	yes	ESI
45	1.5	1.7	-1.5	2.6	2.1	4	no	
46	-1.6	-1.9	-1.9	-1.4	-1.6	5	yes	ESI
47	-1.6	-1.6	-1.7	-1.5	-1.2	5	no	
48	-1.6	-1.5	-2.0	-1.4	-1.6	5	no	
49	-1.5	-1.4	-1.7	-1.6	-1.6	5	no	
50	3.0	1.9	4.0	1.1	-1.4	6	no	
51	-2.0	-1.7	-1.1	1.8	-2.9	6	no	
52	-1.9	1.7	-1.8	-1.4	1.2	6	yes	ESI

Table 2.3 (continued)

53	1.8	2.2	3.7	1.1	-1.2	6	yes	ESI
54	1.8	1.5	2.0	1.3	-1.4	6	no	
55	1.7	-1.1	1.5	-1.1	-1.8	6	yes	ESI
56	1.6	1.6	1.1	2.4	1.4	6	no	
57	-1.6	1.4	-1.8	1.1	1.5	6	yes	ESI
58	1.6	1.6	-1.1	2.0	1.2	6	yes	ESI
59	-1.5	-1.6	-2.1	-1.2	-1.2	6	no	
60	-1.5	-1.6	-1.7	-1.4	-1.2	6	yes	LC
61	1.5	1.9	1.8	-1.1	1.2	6	yes	ESI

[a] Spot volumes were normalized to the following baselines: resistant bulk to susceptible bulk; 588224 late to 588224 5 DPB; 588296, 588484; 588530 resistant time points to the susceptible time points within each genotype.
[b] Expression groups determined as described in the methods and materials.
[c] A successful protein identification (ID) is defined as matching at least one protein to the searched database at the statistical level of "identity" (see methods).
[d] The mass spectrometric method used to determine the protein identity. ESI, Nano-LC-ESI-MS/MS, LC, LC-MALDI-TOF/TOF (see methods).

Because the *in silico* resistant bulk contained only older tissue samples from the OR genotypes, protein spots unrelated to OR were frequently selected (Table 2.3). Fifteen spots were related to ontogenic resistance (Groups 1 and 2), with the remaining spots signifying developmental expression either conserved across genotypes (34) or expressing no consistent pattern (12) (Table 2.3). Those spots which show no consistent pattern (Group 6) suggest traits that exhibit high levels of genotypic variation.

Protein identification. From the 61 spots selected for protein identification (Figure 2.1), 42 (68.9%) yielded identifications. Seventy-three percent of these spots were found to contain more than a single protein (Table 2.4), and 142 proteins were identified, with 85 being unique (Table 2.4). Overall, the average number of proteins identified per spot in this study was 3.4. The identification of multiple proteins in a single 2D gel spot has been observed previously in several other studies [21, 22]. This decreases power in making a correlation of expression of an identified protein and the gain of OR, since a mixture of proteins is influencing the observed fold-change of the spot. A method which involves picking spots from triplicate gels of both the up and down regulated tissues for quantification of each protein member [46] was not a viable approach in this study due to the complexity derived from the time course experimental design and diverse genotypes used. Thus, all proteins identified from a spot of interest must be considered to be potentially correlated to the development of OR.

Table 2.4. Protein identifications organized by functional classification

Spot number[a]	Protein name[b]	Genbank accession number	Total Ion score	Peptides matching	Sequence coverage (%)	Expression group
Regulation						
1	GTP-binding nuclear protein Ran1	gi|585777	61	1	4%	1
2	Hypothetical protein [Vitis vinifera]; Coatomer subunit epsilon-1 (Epsilon-coat protein 1) (Epsilon-COP 1) (Epsilon1-COP) [Oryza sativa (japonica cultivar-group)] gi:75336169	gi|147858975	71	3	21%	1
3	Probable pyridoxal biosynthesis protein PDX1 (Ethylene-inducible protein HEVER)	gi|2501578	54	1	2%	2
3	Hypothetical protein [Vitis vinifera]; PUR ALPHA-1 (purin-rich alpha 1); nucleic acid binding [Arabidopsis thaliana] gi:30685174	gi|147774370	203	11	35%	2
6	14-3-3 protein [Ipomoea nil]	gi|124484407	122	2	9%	2
11	Hypothetical protein [Vitis vinifera]; WD-40 repeat family protein / small nuclear ribonucleoprotein Prp4p-related [Arabidopsis thaliana] gi:15227373	gi|147819065	58	1	1%	2
11	dnaK-type molecular chaperone (clone pMON9508) [Zea mays] (fragment)	gi|283043	63	1	15%	2
11	Hypothetical protein [Vitis vinifera]; Phox (PX) domain-containing protein [Arabidopsis thaliana] gi:22327944	gi|147819587	87	1	8%	2
11	Hypothetical protein [Vitis vinifera]; PAT1 (Phytochrome A signal transduction 1); transcription factor [Arabidopsis thaliana] gi:15238903	gi|147818882	94	3	7%	2
11	Putative transcription factor [Arabidopsis thaliana]	gi|9454552	97	1	2%	2
12	Os07g0614500 [Oryza sativa (japonica cultivar-group)] (Elongation factor 1)	gi|115473331	62	1	3%	2

Table 2.4 (continued)

13	OS04g0270100 [Oryza sativa (japonica cultivar-group)] - GTP-binding protein	gi|115457482	47	1	2%	2
17	Os04g0118400 [Oryza sativa (japonica cultivar-group)] (Elongation factor 2)	gi|115456914	86	10	9%	3
18	Heat shock protein [Glycine max]	gi|530207	53	1	1%	3
18	Os04g0118400 [Oryza sativa (japonica cultivar-group)] (Elongation factor 2)	gi|115456914	132	15	14%	3
20	Protein transport SEC13-like protein [Solanum tuberosum]	gi|83283979	61	1	3%	3
22	Heat shock protein 101 [Vitis vinifera]	gi|59805048	58	1	1%	3
22	Os04g0118400 [Oryza sativa (japonica cultivar-group)] (Elongation factor 2)	gi|115456914	106	11	9%	3
30	GTP-binding nuclear protein Ran1 [Solanum lycopersicum]	gi|585777	54	2	9%	3
32	Hypothetical protein [Vitis vinifera]; 14-3-3-like protein [Pisum sativum] gi:4850247	gi|147805242	131	2	11%	3
33	Hypothetical protein [Vitis vinifera]; RNA recognition motif (RRM)-containing protein [Arabidopsis thaliana] gi:18395106	gi|147802334	57	1	5%	3
33	Hypothetical protein [Vitis vinifera]; Guanine nucleotide-binding protein subunit beta-like protein [Medicago sativa] gi:3023847	gi|147784318	90	2	10%	3
38	Putative transcription factor [Vitis vinifera]; Abscisic stress ripening protein [Vitis pseudoreticulata] gi|86156026	gi|14582465	73	2	14%	4
40	Hypothetical protein [Vitis vinifera]; GUN4, putative [Oryza sativa (japonica cultivar-group)] gi:62734221	gi|147779401	64	1	4%	4
40	Putative transcription factor [Vitis vinifera]; Abscisic stress ripening protein [Vitis pseudoreticulata] gi|86156026	gi|14582465	111	2	14%	4

Table 2.4 (continued)

41	Os01g0667600 [Oryza sativa (japonica cultivar-group)] (putative GTP-binding protein Rab11b)	gi|115439059	54	2	8%	4
46	Hypothetical protein [Vitis vinifera]; Tyrosyl-tRNA synthetase [Nicotiana tabacum] gi:1841468	gi|147815112	57	2	5%	5
46	Hypothetical protein [Vitis vinifera]; RNA recognition motif (RRM)-containing protein [Arabidopsis thaliana] gi:15232459	gi|147789426	66	2	5%	5
46	Hypothetical protein [Vitis vinifera]; ATNAP6 (NON-INTRINSIC ABC PROTEIN 6) [Arabidopsis thaliana] gi:18398463	gi|147866701	71	4	28%	5
52	Os04g0118400 [Oryza sativa (japonica cultivar-group)] (Elongation factor 2)	gi|115456914	112	12	10%	6
57	Hypothetical protein [Vitis vinifera]; Guanine nucleotide-binding protein subunit beta-like protein [Medicago sativa] gi:3023847 (WD-40 domain containing)	gi|147784318	56	1	7%	6
Metabolism						
2	Hypothetical protein [Vitis vinifera]; ADP-sugar diphosphatase [Solanum tuberosum] gi:110622992	gi|147766435	58	1	3%	1
11	Pasticcino 1-D [Arabidopsis thaliana]	gi|3080740	65	1	1%	2
11	L-idonate dehydrogenase [Vitis vinifera]	gi|74273318	74	2	6%	2
11	Hypothetical protein [Vitis vinifera]; Transketolase 1 [Capsicum annuum] gi:3559814	gi|147788852	137	5	5%	2
11	Hypothetical protein [Vitis vinifera]; Transketolase 1 [Capsicum annuum] gi:3559814	gi|147835837	191	11	16%	2
13	Phosphoglycerate mutase (Prunus dulcis)	gi|1498232	97	2	4%	2
18	Hypothetical protein [Vitis vinifera]; Mitochondrial glycine decarboxylase complex P-protein [Populus tremuloides] gi:134142800	gi|147805324	57	1	1%	3
24	Trypsin inhibitor subtype A [Glycine max]	gi|18770	63	2	8%	3

Table 2.4 (continued)

26	Trypsin inhibitor subtype A [Glycine max]	gi|18770	56	1	7%	3
29	Short-chain dehydrogenase/reductase (SDR) family protein [Arabidopsis thaliana]	gi|15233062	46	1	2%	3
32	L-idonate dehydrogenase [Vitis vinifera]	gi|74273318	68	1	2%	3
32	Hypothetical protein [Vitis vinifera] (phosphomannomutase [Triticum aestivum] GI:90762172)	gi|147856872	82	7	21%	3
32	Hypothetical protein [Vitis vinifera] (gamma hydroxybutyrate dehydrogenase-like protein [Oryza sativa (japonica cultivar-group)] GI:29368238)	gi|147861759	85	5	24%	3
33	Malate dehydrogenase, glyoxysomal precursor [Citrullus lanatus]	gi|126894	66	1	4%	3
33	Hypothetical protein [Vitis vinifera]; Putative dTDP-4-dehydrorhamnose reductase [Oryza sativa Japonica Group] gi:48716437	gi|147839168	71	2	5%	3
33	3-desoxy-D-manno octulosonic acid-8-phosphate synthase [Solanum lycopersicum]	gi|13509333	72	1	3%	3
33	Hypothetical protein [Vitis vinifera]; Chloroplast ferredoxin-NADP+ oxidoreductase precursor [Capsicum annuum] gi:6899972	gi|147791392	116	7	16%	3
34	Hypothetical protein [Vitis vinifera]; Putative protein phosphatase 2C [Arabidopsis thaliana] gi:16604585	gi|147855345	101	3	11%	3
38	Hypothetical protein [Vitis vinifera]; Benzoquinone reductase [Gossypium hirsutum] gi:124488474	gi|147788048	104	6	29%	4
39	FQR1 (FLAVODOXIN-LIKE QUINONE REDUCTASE 1) [Arabidopsis thaliana]	gi|15239652	98	3	17%	4
46	L-idonate dehydrogenase [Vitis vinifera]	gi|74273318	66	1	2%	5

Table 2.4 (continued)						
46	GME (GDP-D-MANNOSE 3',5'-EPIMERASE); GDP-mannose 3,5-epimerase/ NAD binding / catalytic [Arabidopsis thaliana]	gi|15241945	99	2	6%	5
55	Hypothetical protein [Vitis vinifera]; Hydroxyphenylpyruvate reductase (HPPR) [Solenostemon scutellarioides] gi:62816284	gi|147843670	107	6	25%	6
57	Hypothetical protein [Vitis vinifera]; Putative dTDP-4-dehydrorhamnose reductase [Oryza sativa Japonica Group] gi:48716437	gi|147839168	111	5	15%	6
60	Transketolase, putative [Arabidopsis thaliana]	gi|30689983	45	1	2%	6
Secondary Metabolism						
3	Hypothetical protein [Vitis vinifera]; cyclase [Vitis pseudoreticulata] gi:86156016	gi|147838052	140	9	16%	2
7	Cyclase [Vitis pseudoreticulata]	gi|86156016	81	2	5%	2
11	Acyl CoA synthetase [Brassica napus]	gi|1617268	80	4	4%	2
11	Hypothetical protein [Vitis vinifera]; AMP binding / acetate-CoA ligase/ catalytic [Arabidopsis thaliana] gi:145323645	gi|147816169	80	1	1%	2
11	Hypothetical protein [Vitis vinifera]; Phenylalanine ammonia-lyase [Camellia sinensis] gi:662271	gi|147801854	177	10	16%	2
13	Phosphoglycerate dehydrogenase-like protein [Arabidopsis thaliana]	gi|7270370	52	1	2%	2
14	Adenylate kinase [Ceratopteris richardii]	gi|5305496	51	1	7%	2
15	Cyclase [Vitis pseudoreticulata]	gi|86156016	171	11	18%	2
20	Hypothetical protein [Vitis vinifera]; Phosphomannomutase [Triticum aestivum] gi:90762172	gi|147856872	223	9	33%	3
24	Hypothetical protein [Vitis vinifera]; Cyclase [Vitis pseudoreticulata] gi:86156016	gi|147838052	57	1	3%	3

Table 2.4 (continued)

24	Polyphenol oxidase, chloroplast precursor (PPO) (Catechol oxidase) [Vitis vinifera]	gi|1172587	82	5	7%	3
25	Polyphenol oxidase [Vitis vinifera]	gi|510234	58	4	7%	3
26	Peptidyl-prolyl cis-trans isomerase (PPIase) (Rotamase) (Cyclophilin) (Cyclosporin A-binding protein) [Catharanthus roseus]	gi|3334157	59	1	6%	3
26	Polyphenol oxidase, chloroplast precursor (PPO) (Catechol oxidase) [Vitis vinifera]	gi|1172587	77	10	11%	3
32	Adenylate kinase [Oryza sativa]	gi|7630193	66	1	4%	3
34	Hypothetical protein [Vitis vinifera]; Cyclase [Vitis pseudoreticulata] gi:86156016	gi|147838052	72	1	3%	3
46	3-hydroxy-3-methylglutaryl-CoA synthase [Taxus x media]	gi|50295909	63	2	4%	5
55	TPA: isoflavone reductase-like protein 4 [Vitis vinifera]	gi|76559892	67	2	3%	6
58	Hypothetical protein [Vitis vinifera]; Polyphenol oxidase, chloroplast precursor (PPO) (Catechol oxidase) [Vitis vinifera] gi:1172587	gi|147766674	59	2	2%	6
58	Polyphenol oxidase [Vitis vinifera]	gi|1785613	59	2	2%	6
60	Os02g0627100 - phenylalanine ammonia-lyase	gi|113537015	65	1	1%	6
61	Chalcone isomerase [Vitis vinifera]	gi|499036	67	2	10%	6
Photosynthesis						
7	Probable oxygen-evolving enhancer protein 2; VvpsbP1 [Vitis vinifera]	gi|37903240	60	1	13%	2
7	Photosystem II oxygen-evolving complex protein 2 [Arabidopsis thaliana] (fragment)	gi|1076373	88	2	92%	2
12	[Segment 5 of 10] Putative oxygen-evolving enhancer protein 1 (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II) (OEC 33 kDa subunit) (33 kDa thylakoid membrane protein) (PS2)	gi|109892873	69	1	100%	2

Table 2.4 (continued)

18	[Segment 5 of 10] Putative oxygen-evolving enhancer protein 1 (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II) (OEC 33 kDa subunit) (33 kDa thylakoid membrane protein) (PS2)	gi|109892873	87	2	100%	3
29	Oxygen-evolving enhancer protein 1-2, chloroplast precursor (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II) (OEC 33 kDa subunit) (33 kDa thylakoid membrane protein)	gi|1134146	44	1	3%	3
37	Hypothetical protein [Vitis vinifera]; Chloroplast chaperonin 21 [Vitis vinifera] gi:50660327	gi|147815877	53	2	8%	4
37	cp10-like protein [Gossypium hirsutum]	gi|21780187	58	1	4%	4
37	Ferritin [Phaseolus vulgaris]	gi|21027	58	1	3%	4
38	Photosystem II oxygen-evolving complex protein 2 - [Arabidopsis thaliana] (fragment)	gi|1076373	54	1	92%	4
40	Hypothetical protein [Vitis vinifera]; 23kDa polypeptide of the oxygen-evolving complex of photosystem II [Cucumis sativus] gi:6691487	gi|147787750	65	2	8%	4
40	Photosystem II oxygen-evolving complex protein 2 [Arabidopsis thaliana] (fragment)	gi|1076373	114	2	92%	4
42	Chloroplast chaperonin 21 [Vitis vinifera]	gi|50660327	88	2	8%	4
42	Hypothetical protein [Vitis vinifera]; Chloroplast chaperonin 21 [Vitis vinifera] gi:50660327	gi|147815877	144	6	23%	4
44	33 kDa polypeptide of oxygen-evolving complex (OEC) in photosystem II [Arabidopsis thaliana]	gi|3286693	133	8	21%	4
61	Putative 33kDa oxygen evolving protein of photosystem II [Oryza sativa (japonica cultivar-group)]	gi|15408655	70	3	12%	6

Table 2.4 (continued)

Proteolysis						
1	Hypothetical protein [Vitis vinifera]; PAG1 (20S proteasome alpha subunit G1); peptidase [Arabidopsis thaliana] gi:15225839	gi|147805763	67	6	20%	1
1	Hypothetical protein [Vitis vinifera]; Proteasome subunit alpha type-6 (20S proteasome alpha subunit A) (20S proteasome subunit alpha-1) gi:12229948	gi|147797489	106	5	18%	1
11	Hypothetical protein [Vitis vinifera]; Xaa-Pro aminopeptidase 1 [Solanum lycopersicum] gi:15384989	gi|147765321	57	3	15%	2
11	Hypothetical protein [Vitis vinifera]; Xaa-Pro aminopeptidase 2 [Solanum lycopersicum] gi:15384991	gi|147766118	82	4	16%	2
11	Hypothetical protein [Vitis vinifera]; Putative serine protease [Populus x canadensis] gi:29786399	gi|147820236	83	1	1%	2
12	Proteasome subunit beta type 1 (20S proteasome alpha subunit F) (20S proteasome subunit beta-6) [Petunia x hybrida]	gi|17380185	63	1	4%	2
19	ATP-dependent Clp protease proteolytic subunit, putative [Arabidopsis thaliana]	gi|18414804	59	3	9%	3
21	Peptidase T1A, proteasome beta-subunit [Medicago truncatula]	gi|92875602	64	2	8%	3
30	Peptidase T1A, proteasome beta-subunit [Medicago truncatula]	gi|92875602	78	1	4%	3
37	Proteasome subunit beta type 1 (20S proteasome alpha subunit F) (20S proteasome subunit beta-6) [Petunia x hybrida]	gi|17380185	78	1	4%	4
40	Proteasome subunit beta type 1 (20S proteasome alpha subunit F) (20S proteasome subunit beta-6) [Petunia x hybrida]	gi|17380185	78	1	4%	4

Table 2.4 (continued)

42	ATP-dependent Clp protease proteolytic subunit, putative [Arabidopsis thaliana]	gi|18414804	60	3	9%	4
42	Proteasome subunit beta type 1 (20S proteasome alpha subunit F) (20S proteasome subunit beta-6) [Petunia x hybrida]	gi|17380185	82	1	4%	4
46	Proteasome component region PCI [Medicago truncatula]	gi|92896846	56	1	2%	5
55	Hypothetical protein [Vitis vinifera]; Putative ubitquitin C-terminal hydrolase [Oryza sativa Japonica Group] gi:48716277	gi|147766506	58	1	3%	6
57	Hypothetical protein [Vitis vinifera]; 26S proteasome non-ATPase regulatory subunit 14 (26S proteasome regulatory subunit rpn11) [Arabidopsis thaliana] gi:51701846	gi|147803561	105	5	18%	6
Respiration						
11	Os06g0499900 [Oryza sativa (japonica cultivar-group)] (putative dihydrolipoamide S-acetyltransferase [Oryza sativa Japonica Group])	gi|115468212	55	3	4%	2
11	Dihydrolipoamide acetyltransferase (E2) subunit of PDC [Arabidopsis thaliana]	gi|559395	58	1	1%	2
17	Putative aconitate hydratase 1 [Sorghum bicolor]	gi|92429669	68	4	3%	3
18	2-oxoglutarate dehydrogenase, E1 subunit-like protein [Arabidopsis thaliana]	gi|7076784	63	1	1%	3
18	Hypothetical protein [Vitis vinifera]; Aconitase (ACO) [Arabidopsis thaliana] gi:15233349	gi|147855123	112	9	11%	3
22	Putative aconitate hydratase 1 [Sorghum bicolor]	gi|92429669	87	4	3%	3

Table 2.4 (continued)

31	Putative aconitate hydratase 1 [Sorghum bicolor]	gi|92429669	45	1	2%	3
46	Hypothetical protein [Vitis vinifera]; NADP-isocitrate dehydrogenase [Eucalyptus globulus] gi:1750380	gi|147826481	84	5	14%	5
50	Hypothetical protein [Vitis vinifera]; ATP synthase D chain, mitochondrial, putative [Solanum demissum] gi:48209968	gi|147818815	57	1	7%	6
52	Putative aconitate hydratase 1 [Sorghum bicolor]	gi|92429669	67	4	4%	6
Stress Response						
1	Ascorbate peroxidase [Vitis pseudoreticulata]	gi|73647738	128	14	38%	1
2	Hypothetical protein [Vitis vinifera]; Aluminum-induced protein-like [Arabidopsis thaliana] gi:10177953	gi|147805616	63	2	8%	1
3	3-mercaptopyruvate sulfurtransferase [Arabidopsis thaliana]	gi|6686778	57	1	2%	2
11	Heat shock cognate protein 80	gi|547683	97	2	3%	2
11	Cell-autonomous heat shock cognate protein 70 [Cucurbita maxima]	gi|26985223	98	5	10%	2
15	Thylakoid-bound L-ascorbate peroxidase precursor [Mesembryanthemum crystallinum]	gi|3202024	67	2	6%	2
32	Ascorbate peroxidase [Spinacia oleracea]	gi|310587	64	2	6%	3
33	Hypothetical protein [Vitis vinifera]; Fiber annexin [Gossypium hirsutum] gi:3493172	gi|147861246	74	8	27%	3
37	Aluminum-induced protein [Codonopsis lanceolata]	gi|56606534	65	1	5%	4
38	Superoxide dismutase [Pisum sativum]	gi|20902	55	1	6%	4
Diesase Resistance						
24	Pathogenesis related protein [Cicer arietinum]	gi|499171	61	1	9%	3
33	Peroxidase, putative [Arabidopsis thaliana]	gi|15237187	57	2	5%	3
36	Pathogenesis related protein [Cicer arietinum]	gi|499171	71	1	9%	4

Table 2.4 (continued)

Other						
11	Hypothetical protein [Vitis vinifera]; P70 protein [Nicotiana tabacum] gi:18076679	gi|147826788	61	3	4%	2
11	Hypothetical protein [Vitis vinifera]; Myosin heavy chain, putative, expressed [Oryza sativa (japonica cultivar-group)] gi:77554602	gi|147822315	62	1	1%	2
24	Hypothetical protein [Vitis vinifera]; Major cherry allergen Pru av 1.0201 [Prunus avium] gi:44409451	gi|147853970	107	3	21%	3
26	Hypothetical protein [Vitis vinifera]; Major cherry allergen Pru av 1.0201 [Prunus avium] gi:44409451	gi|147853970	103	2	21%	3
50	Hypothetical protein [Vitis vinifera]; Pollen allergen-like protein [Arabidopsis thaliana] gi:86156016	gi|147776917	70	4	29%	6
53	Hypothetical protein [Vitis vinifera]; Pollen allergen-like protein [Arabidopsis thaliana] gi:21593946	gi|147776917	63	2	16%	6
Unknown						
20	Hypothetical protein [Vitis vinifera]; unknown protein	gi|147765951	68	6	23%	3
29	Putative protein [Arabidopsis thaliana]	gi|7270078	52	1	3%	3
57	Hypothetical protein [Vitis vinifera]; unknown protein	gi|147792793	108	1	6%	6
61	Conserved hypothetical protein [Medicago truncatula]	gi|92871033	60	1	3%	6

[a] Spot number listed in Table 2.3.

[b] Sequence title including organism of the protein identified from a MASCOT search of Viridiplantae sequences in the NCBInr database (January, 2008). The top hit (highest score) was provided when homologous proteins were identified. When a Hypothetical protein [Vitis vinifera] was matched, an annotated match is provided as described in the methods and materials.

[c] The summation of peptide scores above the identity threshold.

Two different ionization methods were used in this study: LC-ESI-MS/MS and LC-MALDI. LC-ESI-MS/MS identified at least one protein in 36 out of 40 spots tested. The LC-MALDI technique was successful in identifying at least one protein in six out of 21 spots. LC-ESI-MS/MS found 27 spots containing multiple proteins (75.0%) and LC-MALDI identified multiple proteins in three spots (50.0%). The higher rate of success in identifying proteins from trypsin-digested gel spots using LC-ESI-MS/MS over LC-MALDI was demonstrated in a previous study [46].

Of the proteins identified, 59 (41.5%) had only a single matching peptide (Table 2.3). Since the statistical confidence of a single peptide match is lower than multiple matches, these "one-hit wonders" imbue a lower confidence in the identification. Still, all peptides considered in this study had an ion-score in MASCOT greater than or equal identity cut-off threshold. Using the decoy database option in Mascot gave a false-discovery rate of 1.9%, suggesting that the overall set of protein identifications can be interpreted with a high level of confidence that nearly all of the one-hit wonders provided correct identifications. For all 142 proteins identified, the average number of corresponding peptides was 3.06 and was 4.52 when excluding the identifications with only single matching peptide.

Some spots from different areas of the gel were found to contain the same identified protein, which could be due to post-translational modifications, sequence variation of homologous genes or allelic variation of the orthologous protein from the different genotypes (Figure 2.1). This phenomenon has been seen in other proteomic studies [21, 22, 46]. This observation can provide additional information (besides spot fold-change and protein identification) about the biological system being studied, and remains of interest for future investigations.

The inclusion of the draft *Vitis vinifera* genome [19] in the database used for MASCOT searches made it possible to use the peptide information from this study to annotate 55 hypothetical proteins for function (Table 2.4). The annotations were made by comparing matched peptides of the hypothetical proteins to proteins of known function in the same match. A follow-up search using BLASTP [1] was used to increase the confidence of the match. This demonstrates an additional value of proteomic studies as a means of giving high-confidence annotations to a draft genome.

Oxidation analysis to resolve ambiguous residue identity. The analysis of spot 13 (Table 2.4) yielded an ion at M/Z 942.4567 which could not be unambiguously assigned as two peptide sequences that were consistent with the precursor mass and fragmentation pattern. The two nearly isobaric sequences were IYEGEGFK, (M_r = 942.4573) and IYEGEGM^{o}K (M_r = 942.4293), each corresponding to a different match in NCBInr. The deviation of the measured mass and the predicted masses for these ions was within experimental error and therefore we could not distinguish between the two possibilities. Thus, the MALDI spot from which this ion was observed was subjected to peracid oxidation as has been previously described [45]. If the ion responsible for the 942.4567 peak contained a M^o oxidation, peroxide oxidation would convert it to M$^o_2$, resulting in an observed mass shift of 16 Da. After oxidation, no mass shift was observed, and we concluded that the correct sequence is IYEGEGFK, with the best match being Phosphoglycerate mutase (*Prunus dulcis*) (Table 2.4).

Evaluation of phenotypic bulking for working with a high level of genetic variation. Using disparate host genotypes in a phenotypic bulking of protein extracts was successful for investigating insect resistance in rice [31]. We adapted this

strategy to study protein expression relating to OR in grapevine. We used four genotypes of *Vitis spp.* that exhibited categorized phenotypes. This method delivered promising results by identifying a small number of spots-of-interest.

However, there were some technical limitations with this approach that lowered the precision of the expression analysis. Primary among these is the identification of multiple proteins from a single gel spot. Any one or more of the proteins in the spot could have been responsible for the observed, significant fold-change. As described above, another potential issue is the inter-genotype allelic variation in protein sequence sufficient to cause migration differences. This would make it difficult to correlate the expression profile of a protein using spot fold-change due to the possibility of orthologous proteins not co-migrating.

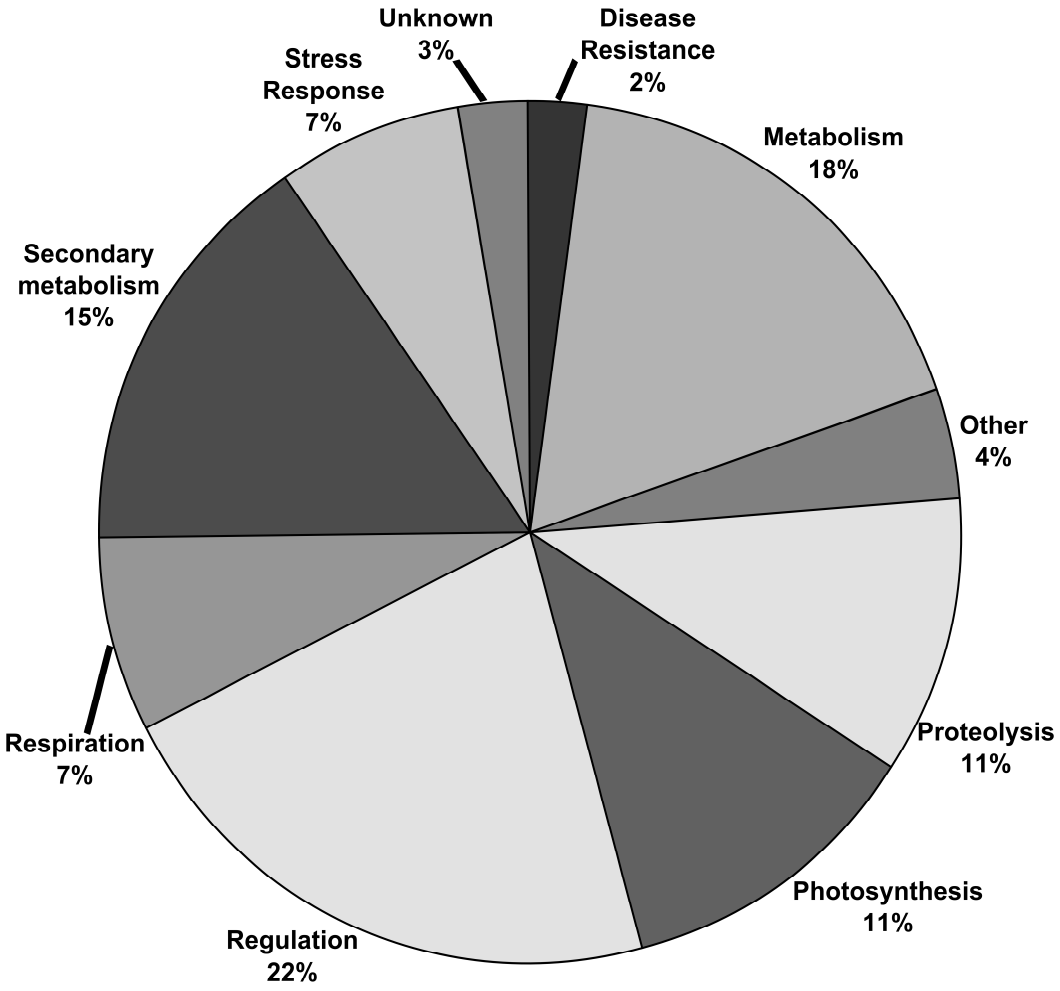
A method using triplicate picking gels of individual genotype/time-point combinations (see [46]) to select every spot for trypsin-digestion and identification would give information on the migration of individual proteins from each genotype, which could be used to track orthologous proteins with different migration patterns. This in turn could be used to make more accurate determination of specific protein activity and to investigate which orthologs are most important. However, this strategy would result in very large numbers of protein gels and mass spectrometry analysis (4 genotypes with at least 2 time points and run in triplicate (24 gels) x 1000 spots x 20 fractions = 480,000 MALDI Spots), which would have been beyond available resources. Still, for both analyses, protein quantification is of individual spots instead of the individual proteins. In the end, the *in silico* bulking method used in this study was successful in narrowing down the list of spots of interest to a manageable number of 15, which included 43 proteins (Table 2.4).

Protein expression in grape berry skins during early berry development. This study provided insights into the development of grape berries during the first 30 days following anthesis. Focusing on this period makes this study one of the few to investigate early berry development at the protein level, and the only known to focus on the first month of development following anthesis [5, 16]. This study also provides information related to early development of grape skins, helping improve understanding of development in this important, non-climacteric fruit system.

Proteins identified were categorized into several functional classes (Table 2.4, Figure 2.2). Broadly, these classes cover many aspects of berry development and growth. Gene regulation and metabolism had the most identified proteins, with secondary metabolism, photosynthesis and proteolysis machinery also containing many members (Table 2.4, Figure 2.2). Less-represented groups are involved in stress response and disease resistance (Table 2.4, Figure 2.2). Several proteins were correlated to the development of berries, which can aid not only the study of OR, but also studies of other aspects of early berry physiology. These results likely reflect the biologically active time-period of early berry development in grapevine [4]. These patterns have also been observed in another proteomic study of grape berries [16].

During early berry development, representing the first half of a double-sigmoidal growth pattern, berry growth is primarily controlled by rapid cell division and cell expansion [15]. As have been shown in previous studies of early grape berry development [5, 16], this growth is driven by proteins involved in metabolic and photosynthetic pathways.

Figure 2.2. A pie-chart depicting the ratio of functional classification of identified proteins listed in Table 2.4.


Unknown
3%
Disease
Resistance
2%
Stress
Response
7%
Metabolism
18%
Secondary
metabolism
15%
Other
4%
Respiration
7%
Proteolysis
11%
Photosynthesis
11%
Regulation
22%

This has been demonstrated here by expression of proteins such as phosphoglycerate mutase, polyphenol oxidase and the 33-kDa subunit of the oxygen evolving complex (Table 2.4). Finding similar pathways in the present study not only bolsters previous investigations of grape berry development, but also broadens our understanding of *Vitis* as novel and diverse genotypes were used.

By using phenotypic bulking, and selecting proteins whose expression correlates to resistant tissue, selected spots are associated with the gain of resistance. The fact that from these spots, only three proteins were classified as disease resistance and 10 related to stress response (see above) might indicate the mode of action of OR is driven by factors involved in berry development, rather than those that can be classified as part of an active defense against pathogen attack. That so few proteins typical of host plant disease defense were identified here might explain why previous efforts to discover the molecular basis for OR, focused on pathogenesis-related proteins, were not successful in finding a significant pattern of expression correlated to the gain of resistance [9]. Low level accumulation of disease resistance and stress response proteins in grape berries has been shown in previous studies [5, 16], while another has shown these proteins concentrate toward maturity [34].

Proteasome activity was previously implicated in basal resistance, a component of innate immunity, against barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) (*Bgh*) [6]. Cells transformed using transient induced gene silencing to cause a partial reduction in cellular ubiquitin levels showed dramatically increased host susceptibility to *Bgh* when compared to control cells [6]. Ubiquitin-related expression was also elevated in EST libraries from immature green berries [39]. Several proteasome subunits were identified in this study, including in the expression groups 1 and 2

(Table 2.4). These findings allude to possible connections between OR and basal resistance, offering targets for further analysis. Another protein of interest that showed elevated expression in OR tissue is ascorbate peroxidase (EC 1.11.1.11), whose over-expression was shown to increase resistance in Tobacco (*Nicotiana tabacum* cv. xanthi) to the hemibiotrophic oomycete *Phytophthora nicotianae* [33]. Further, ascorbate peroxidase was implicated in enhanced resistance in tomato to biotrophic pathogens [32] and to necrotropic *Botrytis cinerea* in detached tomato leaves [23].

There was a clear pattern of protein expression related to photosynthesis during early berry development. Photosynthesis also emerged during the same developmental time-period at the transcript level [39, 43] and when quantified by chlorophyll content [28]. These findings are of interest because the main contributor to sugar accumulation during veraison has been attributed to import from the canopy via the phloem [38]. This study suggests that photosynthesis during early development might have a role to play in development.

In this study, 2D proteomic techniques have been successfully deployed to further understand the breadth and dynamics of grape berry development. These strategies however, can be complicated when attempting to use unrelated germplasm, which for the study of OR [15] was the only option currently available. Our effort to overcome this obstacle by using *in silico* phenotypic bulking of protein samples was successful in narrowing the list of spots of interest, although was not powerful enough to quantify expression of individual proteins within those spots. Furthermore, this study was successful in providing interesting information about the earliest stages of development in grape berries. This information will serve as the foundation for future

physiological investigations of grape development, including those that will provide further insight into the development of ontogenic resistance.

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CHAPTER 3

Chemical and physical analysis of the berry epicuticular surface of *Vitis spp.* during development concomitant with the ontogenic gain of resistance to powdery mildew (*Uncinula necator*)

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ABSTRACT

Wax chemistry and surface topology of developing grape berry cuticles were investigated to determine if they were factors in the development of ontogenic resistance to powdery mildew (*Uncinula necator*). We used three genotypes characterized for their pattern of susceptibility to powdery mildew: (i) constitutive resistance (CR), (ii) ontogenic resistance (OR), and (iii) susceptible (SU) throughout the growing season. Oleanolic acid was the major constituent of the cuticle of all genotypes during the first month of berry development. Proportions of all other cuticle waxes changed during development, with tetracosanal most implicated for involvement in the development of ontogenic resistance. The cuticle surface changed from distinct ridges to amorphous and flat in developing berries of the CR and OR genotypes, while the SU genotype retained significant surface structure composed of the cuticular and minor ridges typical of juvenile berries. Similarly, berries stunted due to lack of seed development remained susceptible and their cuticular topology remained in a state which appeared to be juvenile throughout the growing season. Collectively, our results suggest a possible mechanism for ontogenic resistance that involves both chemical and topographical changes in cuticular waxes during berry development.

INTRODUCTION

By forming a barrier between the environment and interior tissues, plant cuticles limit water loss, function in gas exchange, and provide defense against some pests and pathogens [24]. The cuticle comprises of a structural layer of cutin, (C_{16} to C_{18} hydroxy-fatty acid esters [28] with interspersed intracuticular waxes) and an external

surface covering of epicuticular waxes [1]. The structure and chemical composition of the cuticle on grapevine (*Vitis spp.*) berries have been investigated in several studies [5, 20-24, 29], most of which focused on European grapevine (*V. vinifera*). These investigations have shown that grape berry cuticles are composed of a mixture of complex aliphatic hydrocarbons, highly enriched for triterpenoid acids, and possess extensive surface structure. Also shown was that the cuticle undergoes extensive physical and chemical development as the berries age, with the mature cuticle appearing quite distinct from that at bloom and fruit set.

The berry cuticle is also the site of interaction with the obligate biotroph *Uncinula necator* (Schwein.) Burrill (Syn: *Erysiphe nector*), the causal agent of grapevine powdery mildew, a highly-destructive pathogen occurring in all major grape growing regions of the world [19]. The pathogen infects the host by direct penetration through the cuticle and parasitizes individual epidermal cells for the assimilation of photosynthates [25].

The susceptibility of grape berries to powdery mildew occurs in a limited window of approximately two to three weeks [8, 10-12, 26], which is concomitant with chemical and structural changes in the cuticle [5]. This developmentally-regulated ontogenic resistance (OR) reduces the ability of the pathogen to penetrate and form a haustorial feeding structure in host epidermal cells [8-11], making the cuticle a candidate in the mechanism of OR.

However, the observation of increasing cuticle thickness during this first 2-3 weeks of berry development was shown not to be the mechanism of resistance [7]. This suggests that if the cuticle is involved in OR-based penetration resistance, more subtle

aspects of the cuticle may be involved. Therefore, the current study was carried out to determine if developmental changes in cuticle chemistry or topology are responsible for the development of OR.

METHODS AND MATERIALS

Vineyard setup and tissue collection. Three genotypes of 20-year-old, field-grown vines at the USDA-ARS cold-hardy *Vitis* germplasm collection located in Geneva, New York were used for these experiments. Included was *V. hybrid* 'Red Amber' (PI: 588296) which exhibited a typical ontogenic resistance (OR); *V. rupestris* 'R-65-44' (PI: 588224), which remained susceptible (SU) throughout the growing season; and *V. hybrid 'T*om's Favorite' (PI: 483176) whose berries were resistant throughout development (constitutive resistance, CR) [12]. Shot berries from *V. hybrid 'T*om's Favorite' were separated from the normally developing berries and prepared separately for all analyses. Plant introduction (PI) numbers correspond to the individual genotype records found in the GRIN database [27]. Each genotype was represented by two adjacent, replicate, own-rooted vines planted on 1.8 m centers using a three-wire trellis, cane-pruned and trained using the Umbrella Kniffin system.

Cuticle extraction and GC-FID, GC/MS analysis. Clusters for cuticle extraction were monitored for bloom (defined as 50% anthesis) and randomly assigned a collection time point of 7, 15, 21 or 28 days post bloom (DPB). Two clusters were selected for each time point to act as biological replicates. Following collection, clusters were immediately placed over ice, transported to the laboratory, and maintained at 4°C until use for cuticle wax extraction. Prior to extraction, berries were removed from the rachis, taking care to keep the pedicel intact. Normally

developing berries and shot berries, or berries stunted in growth and development [6], were separated and handled separately for analysis. Cuticular waxes were removed by dipping individual berries into chloroform continuously for 15 s. Berries from a single cluster were dipped in the same beaker to pool all wax from the entire cluster. The chloroform extractions were dried under N_2-flux and stored at -20° C under N_2 until analysis.

The dried wax extractions were dissolved in 2 ml of chloroform and warmed to 50° C to promote solubilization. Hexadecane (5 μg) was spiked in each sample as an internal standard for elution position and peak volume determination. Vials were dried under N_2-flux at 50° C and subsequently dissolved in 100 μl of N,O-Bis(trimethylsilyl)trifluoroacetamide with 1.0% trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO) followed by incubation at 100° C for 15 min. Sample volume was corrected to 250 μl by the addition of hexane. Samples in sealed ampules were directly used for GC analysis and stored at -20° C following injection.

Chromatographic separation, adopted from Chen *et al.* [4], was carried out using a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with a flame-ionization detector (FID) (Palo Alto, CA). The GC-FID was equipped with an 11 m x 0.2 mm HP-1 capillary column with helium as the carrier gas. The GC was set with a starting temperature of 80° C which increased at a rate of 15° C/min to 260° C, followed by a 10 min isothermic period, and finishing with a temperature increase of 5° C/min to a final temperature of 320° C held for 15 min.

Quantification was based on FID peak areas relative to the hexadecane internal standard. Correction factors for groups of compounds known to be present in grape

berry epicuticular waxes (*e.g. n*-alkanes, primary fatty alcohols, aldehydes, long-chain fatty acids) served as the authenticated standards and were applied to the peak areas as described by Jenks *et al.* [14, 15]. Components of the wax extractions were identified by comparison of elution time to the aforementioned external standards. Mass spectra obtained for select samples using a GC-MS (FinniganMAT/Thermospray Corp., San Jose, CA) were used to verify the elution position and elution order for all identified peaks.

The quantity of wax components was expressed as mass per unit berry surface area. Berry surface area was determined by measuring the diameter of 10 representative berries prior to cuticle extraction and using the calculated average diameter to solve for area of a sphere ($A=\pi d^2$). This average surface area was multiplied by the total number of berries extracted to give the total extracted surface area. Quantifications for each identified peak ($\mu g/mm^2$) were averaged across biological replicates and standard deviation was calculated in Excel (Microsoft Corporation, Redmond, WA.).

For analysis of the relative representation of chemical classes, peaks were grouped by chemical class and transformed from the measured quantity to a percentage of its relative contribution to the chemical class. Triterpenoids were not included in this latter analysis due to the lack of multiple chain lengths in this class.

SEM sample preparation and imaging. Berries were harvested at assigned time points (7, 15, 21, or 28 days post bloom) from clusters selected for wax extraction and GC analysis. Hand-sectioned berry peels were collected using a double-edged razor blade and fixed overnight either in 1.0% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) or in 3.125% glutaraldehyde prepared in a 0.1 M cacodylate buffer

(Electron Microscopy Sciences, Hatfield, PA) at RT. Samples fixed in glutaraldehyde were kept under vacuum at -70 kPa during fixation.

Following fixation, berries were washed twice in deionized water, dehydrated with an increasing ethanol series (10%, 20%, 40%, 80%, 100%, 100% for at least 15min each step at RT), and stored in 100% ethanol at 4° C until further use. Samples were dried using a Bal-Tec CPD-030 critical point dryer (Bal-Tec, Balzars Liechtenstein), affixed to aluminum stubs using double-sided tape, and coated with gold using a Balzers SCD-040 sputter coater (Bal-Tec, Balzars Liechtenstein), operated at 60 mA for two coatings of 1 min. Stubs were examined using a Hitachi S-530 scanning electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) (Pleasanton, CA) with a 20 kV acceleration and imaged using Polaroid Type 55 film (Polaroid Corporation, Waltham, MA).

RESULTS

Cuticular wax analysis. For all genotypes, the cuticles from normally developing (non-shot) berries were predominately composed of the triterpenoid oleanolic acid (OA) at all time points (Table 3.1). The minor constituents of the cuticle extractions (*i.e.* acids, aldehydes, alkanes and alcohols) varied in quantity relative to each other depending on genotype and time point (Table 3.1). Through development, chain length bias exhibited a general shift toward longer chained molecules in each chemical class (Figure 3.1).

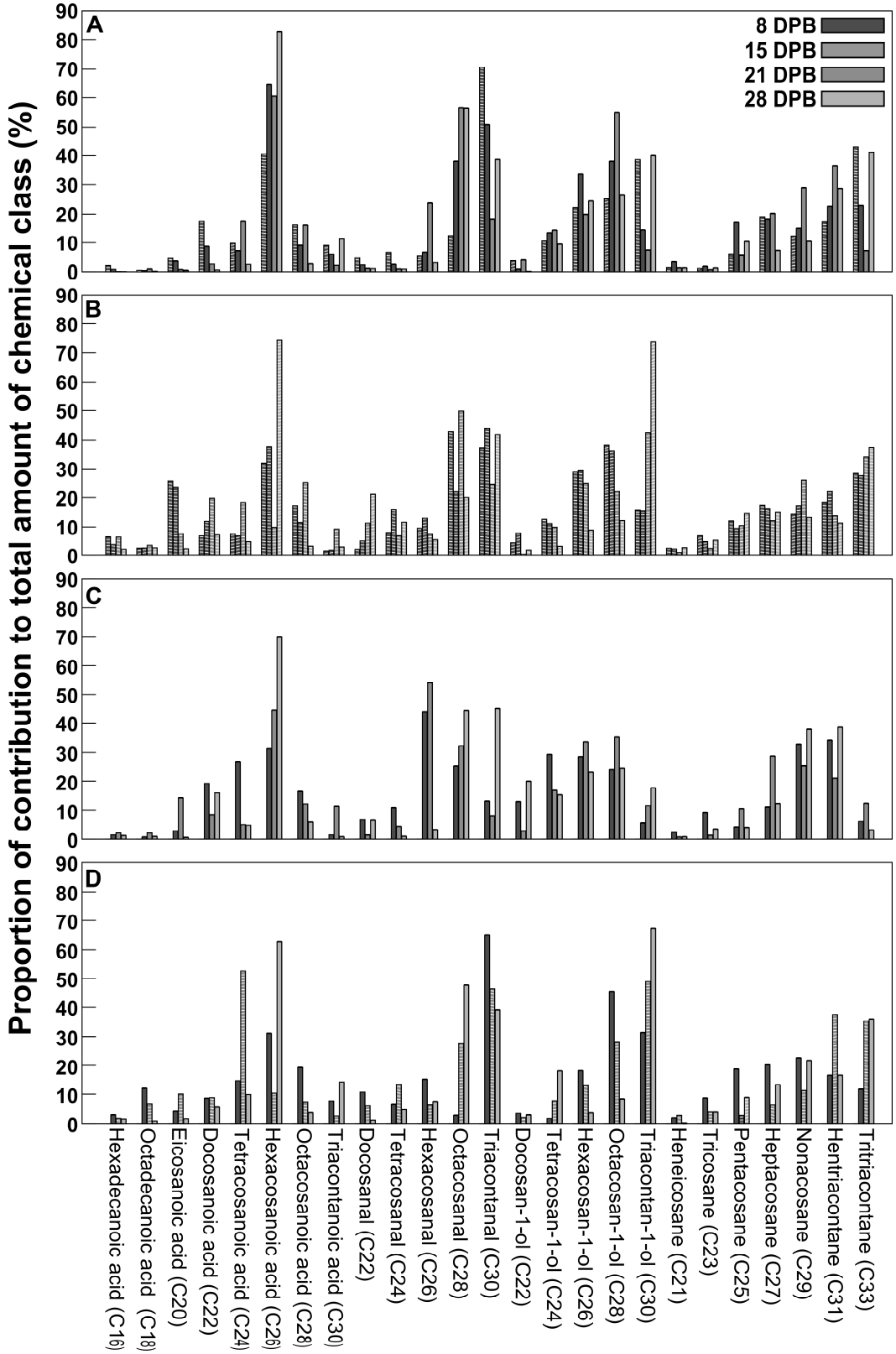
Shot berries from *V. hybrid* 'Tom's Favorite' (PI: 483176) had a cuticle composition distinct from that of normally developed berries from the same clusters (Table 3.1).

Table 3.1. Relative representation of each chemical class identified in grape berry cuticle extractions, by *Vitis* genotype and developmental stage.

Genotype[a]	DPB[b]	(%)								
		Acids[c]		Aldedydes[c]		1° Alcohols[c]		*n*-Alkanes[c]		Triterpenoids[c]
V. hybrid 'Tom's Favorite'	8	7.3 (n/a)	21.2 (n/a)	13.3 (n/a)	38.6 (n/a)	8.9 (n/a)	25.8 (n/a)	5.0 (n/a)	14.5 (n/a)	65.6 (n/a)
	15	2.0 (0.1)	17.8 (1.0)	4.5 (0.2)	39.2 (1.5)	4.1 (0.6)	35.9 (2.7)	0.8 (0.1)	7.1 (0.1)	88.5 (0.9)
	21	3.2 (2.2)	44.7 (16.2)	1.9 (0.9)	27.2 (3.5)	1.2 (0.3)	20.1 (12.5)	0.4 (0.3)	7.9 (7.3)	93.4 (2.5)
	28	7.1 (1.5)	43.0 (11.7)	4.1 (1.5)	24.0 (7.4)	4.9 (0.7)	29.0 (2.8)	0.7 (0.3)	4.0 (1.5)	83.3 (1.0)
V. hybrid 'Tom's Favorite' shot berry[d]	8	20.7 (n/a)	25.9 (n/a)	32.2 (n/a)	40.4 (n/a)	19.1 (n/a)	24.0 (n/a)	7.7 (n/a)	9.7 (n/a)	20.3 (n/a)
	15	29.9 (n/a)	37.5 (n/a)	20.4 (n/a)	25.5 (n/a)	18.2 (n/a)	22.8 (n/a)	11.3 (n/a)	14.2 (n/a)	20.3 (n/a)
	21	4.6 (n/a)	7.7 (n/a)	11.6 (n/a)	19.2 (n/a)	40.7 (n/a)	67.5 (n/a)	3.4 (n/a)	5.7 (n/a)	39.6 (n/a)
	28	26.8 (n/a)	36.8 (n/a)	13.0 (n/a)	17.8 (n/a)	29.5 (n/a)	40.5 (n/a)	3.6 (n/a)	4.9 (n/a)	27.2 (n/a)
V. hybrid 'Red Amber'	15	2.0 (0.5)	33.3 (15.8)	1.9 (0.7)	31.7 (4.1)	1.6 (1.2)	25.0 (14.6)	0.6 (0.0)	10.0 (2.9)	93.8 (1.3)
	21	2.2 (1.2)	30.0 (24.1)	5.9 (4.8)	45.6 (19.2)	2.1 (1.7)	15.6 (6.9)	0.9 (0.2)	8.9 (2.0)	88.9 (5.5)
	28	7.0 (0.5)	59.7 (1.8)	1.8 (1.3)	16.0 (10.4)	1.4 (1.0)	10.9 (8.8)	1.6 (0.3)	13.5 (3.4)	88.1 (0.5)
V. rupestris 'R-65-44'	15	3.4 (0.2)	28.6 (2.5)	2.9 (0.8)	26.0 (7.7)	4.4 (1.4)	34.5 (9.7)	1.4 (0.1)	10.9 (0.4)	87.9 (0.5)
	21	9.7 (n/a)	45.3 (n/a)	2.9 (n/a)	13.6 (n/a)	6.4 (n/a)	29.9 (n/a)	2.4 (n/a)	11.2 (n/a)	78.7 (n/a)
	28	1.8 (0.1)	38.3 (7.7)	1.3 (0.2)	27.7 (8.2)	0.8 (0.5)	17.0 (8.1)	0.8 (0.5)	17.0 (7.7)	95.2 (0.7)

[a] Genotypes listed are taken from the GRIN database [27]. *Vitis* interspecific hybrids are listed as *V. hybrid*.
[b] Days post bloom.
[c] Values in parentheses represent one standard deviation of the mean when available, n/a used when standard deviation is not available. The first column in each chemical grouping represents the average contribution of each chemical class compared to the total wax extraction with the second column representing the average contribution to the total wax fraction not including triterpenoids.
[d] Shot berries are those that remain stunted in growth and development throughout the growing season [6].

Figure 3.1. Graphs showing relative representation of each chain length of the chemical classes: long-chain fatty acids, aldehydes, primary fatty alcohols and *n*-alkanes for genotypes (A) *Vitis hybrid* 'Tom's Favorite' (PI: 483176); (B) shot berries from *V. hybrid* 'Tom's Favorite'; (C) *V. hybrid* 'Red Amber' (PI: 588296); and (D) *V. rupestris* 'R-65-44' (PI: 588224). Error bars represent one standard deviation. Data bars drawn with cross-hatching represent unreplicated samples shown with no error bars. Time points listed are in days post bloom (DPB).


Proportion of contribution to total amount of chemical class (%)
A
B
C
D
8 DPB
15 DPB
21 DPB
28 DPB
0
10
20
30
40
50
60
70
80
90
Hexadecanoic acid (C16)
Octadecanoic acid (C18)
Eicosanoic acid (C20)
Docosanoic acid (C22)
Tetracosanoic acid (C24)
Hexacosanoic acid (C26)
Octacosanoic acid (C28)
Triacontanoic acid (C30)
Docosanal (C22)
Tetracosanal (C24)
Hexacosanal (C26)
Octacosanal (C28)
Triacontanal (C30)
Docosan-1-ol (C22)
Tetracosan-1-ol (C24)
Hexacosan-1-ol (C26)
Octacosan-1-ol (C28)
Triacontan-1-ol (C30)
Heneicosane (C21)
Tricosane (C23)
Pentacosane (C25)
Heptacosane (C27)
Nonacosane (C29)
Hentriacontane (C31)
Tritriacontane (C33)

At all time points, shot berry cuticles had significantly less OA than normally developed berries did. Aside from OA, shot berry cuticles also had variation in the predominate component of the cuticle, including a higher level of fatty acids at 15 DPB and a higher accumulation of primary alcohols between 21 and 28 DPB. Chain length preference, however, was similar to that of the normally developing berries (Figure 3.1).

SEM. The topology of the berry cuticle exhibited extensive structural reconfiguration during development in all genotypes observed (Figures 3.2-4). The earliest time point (7 DPB) was typified by the presence of distinct cuticular ridges, also known as vermicular ridges [5]. These densely packed ridges were approximately 1.5 μm in width (Figures 3.2A, 3.3A, 3.4A).

Change in surface topology was seen as early as 15 DPB, when the cuticular ridges became interspersed with shallower minor ridges, approximately 0.3 μm in width (Figures 3.2B, 3.3B, 3.4B). At 21 DPB, divergence in structure between genotypes was observed, with the CR and OR genotypes beginning transition to a smoother surface, with cuticular ridges becoming rather sparse (Figures 3.2C, 3.3C). The SU genotype lacked the surface smoothing seen in the other two genotypes (Figure 3.4C). The 28 DPB time points in the CR and OR genotypes (Figures 3.2D, 3.3D) showed nearly complete flattening of the cuticle into broad wax platelets, while the SU genotype's cuticle remained almost exclusively minor ridges (Figure 3.4D).

Figure 3.2. Scanning electron microscopy images depicting cuticle surface development of *Vitis hybrid* 'Tom's Favorite' (PI: 483176), an interspecific hybrid that exhibits constitutive resistance of berries during development. Time points displayed are (A) 7 days post bloom, (B) 15 days post bloom, (C) 21 days post bloom and (D) 28 days post bloom. A rapid change in the surface topology of the berry can be seen with the vermicular ridges seen in the earliest development point becoming covered with new layers of deposition until the surface progresses to a nearly smooth surface by 28 days post bloom. The scale bar represents 10 μm.

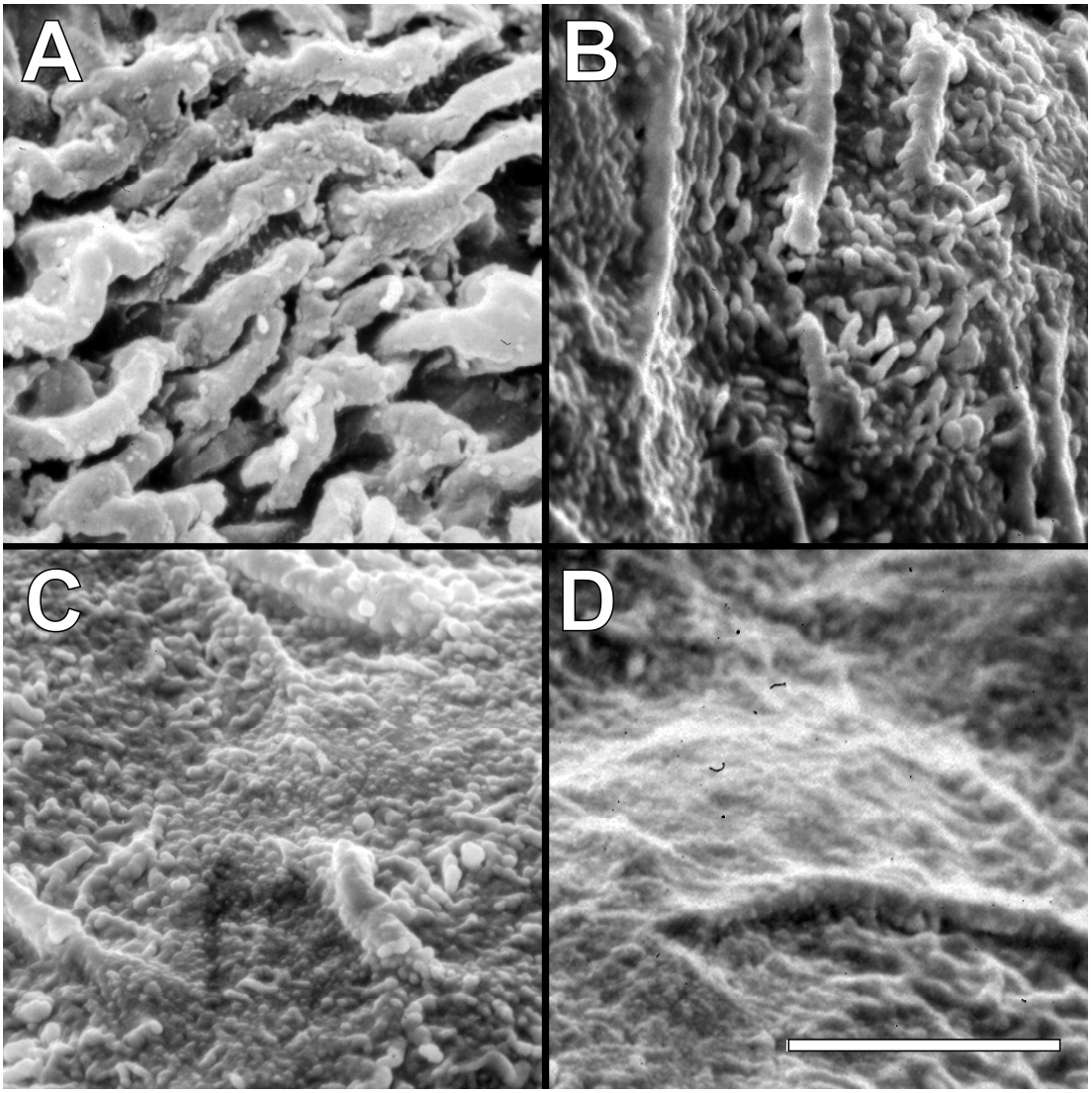

A
B
C
D

Figure 3.3. Scanning electron microscopy images depicting cuticle surface development of *Vitis hybrid* 'Red Amber' (PI: 588296), an interspecific hybrid that develops ontogenic resistance on berries during development. Time points displayed are (A) 7 days post bloom, (B) 15 days post bloom, (C) 21 days post bloom and (D) 28 days post bloom. A rapid change in the surface topology of the berry can be seen with the vermicular ridges seen in the earliest development point becoming covered with new layers of deposition until the surface progresses to a nearly smooth surface by 28 days post bloom. The scale bar represents 10 μm.

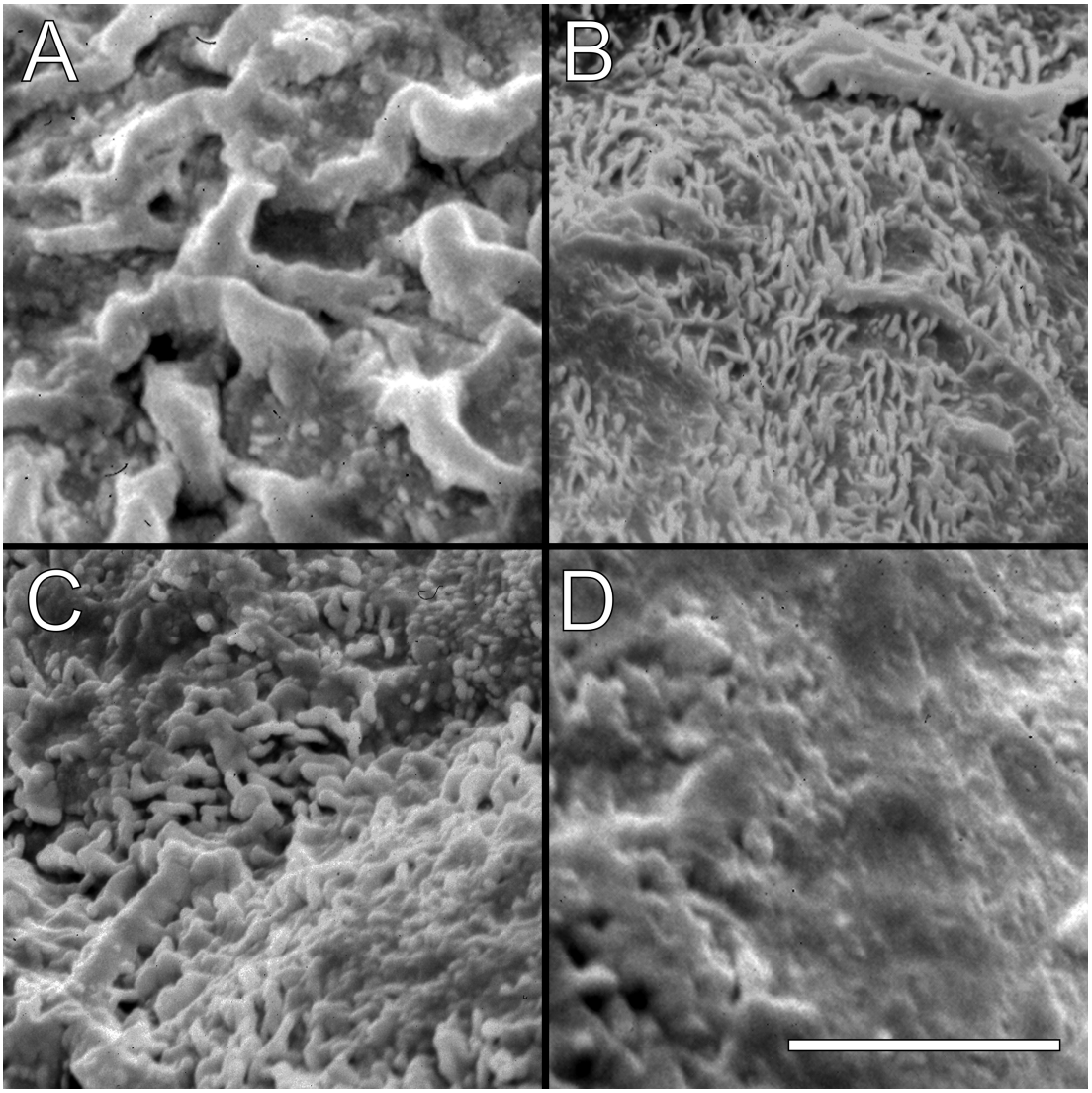
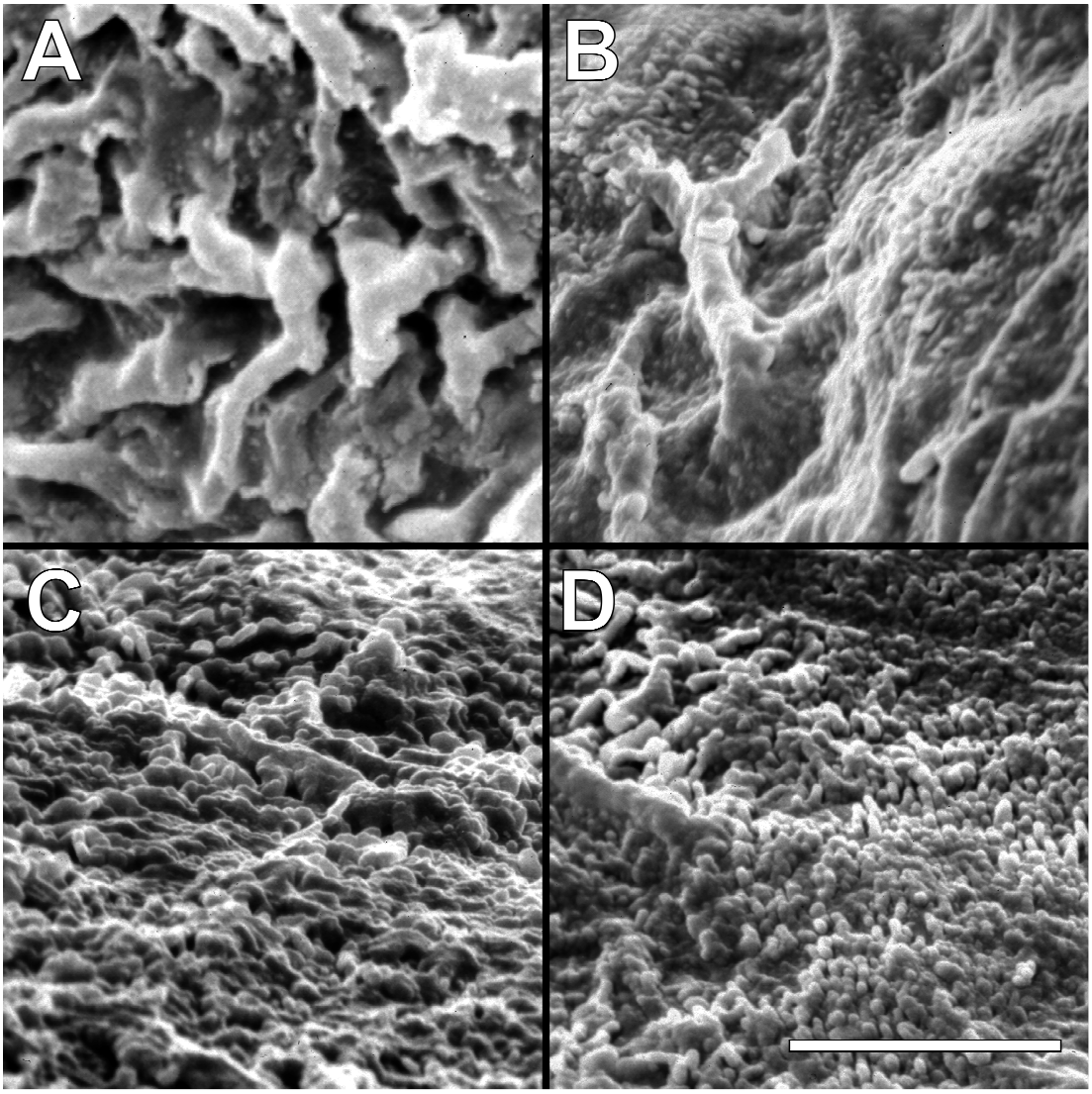

A
B
C
D

Figure 3.4. Scanning electron microscopy images depicting cuticle surface development of *Vitis rupestris* 'R-65-44' (PI: 588224), a wild-collected accession that fails to gain ontogenic resistance on berries during development. This genotype is the only one found to date to maintain susceptibility to *Uncinula necator* during development (2). Time points displayed are (A) 7 days post bloom, (B) 15 days post bloom, (C) 21 days post bloom and (D) 28 days post bloom. A rapid change in the surface topology of the berry can be seen with the cuticular ridges observed in the earliest development point becoming covered with new layers of deposition with the surface becoming highly restructured until becoming covered with smaller granules and ridges. The scale bar represents 10 μm.


A
B
C
D

The cuticle structure of shot berries (PI: 483176) changed little over time, remaining a mixture of cuticular ridges and minor ridges (Figure 3.5), most similar to the SU cuticle. Examples of shot berries from an otherwise mature cluster (71 DPB) also appeared similar to normal, juvenile berries at approximately 4-7 DPB (data not shown).

Functioning stomata were found on the surface of berries of *V. hybrid* 'Red Amber' (PI: 588296) and *V hybrid* 'Tom's Favorite' (PI: 483176) (Figure 3.6). The stomata of the 28 DPB berries appear to be covered in deposits of wax (Figure 3.6C).

DISCUSSION

Cuticle chemistry during early berry development. The cuticular chemistry of the selected genotypes exhibited change during the first month of development, with the triterpenoid OA comprising the largest fraction at all time points in all genotypes (Table 3.1). The levels of OA between 15 and 28 DPB were much higher in the clusters in this study than found in previous studies, where concentrations were reported to be between 20-35% of total cuticular waxes [2, 21]. Other studies demonstrated levels of OA similar to the present study in various grape varieties, although from later developmental time points [13, 20, 29]. Previously, the level of OA was shown to accumulate during development reaching maximum levels at maturity [5], while in this study, the levels were similar in normally developing berries at all time points starting as early as 8 DPB through the conclusion of sampling at 28 DPB (Table 3.1).

Figure 3.5. Shot berries from the constitutively resistant genotype *V. hybrid* 'Tom's Favorite' (PI: 483176) showing stunted development of the cuticular surface with ridges typical of an early time point in development persisting through all points shown. Time points shown are (A) 15 days post bloom, (B) 21 days post bloom and (C) 28 days post bloom. The scale bar represents 10 μm.

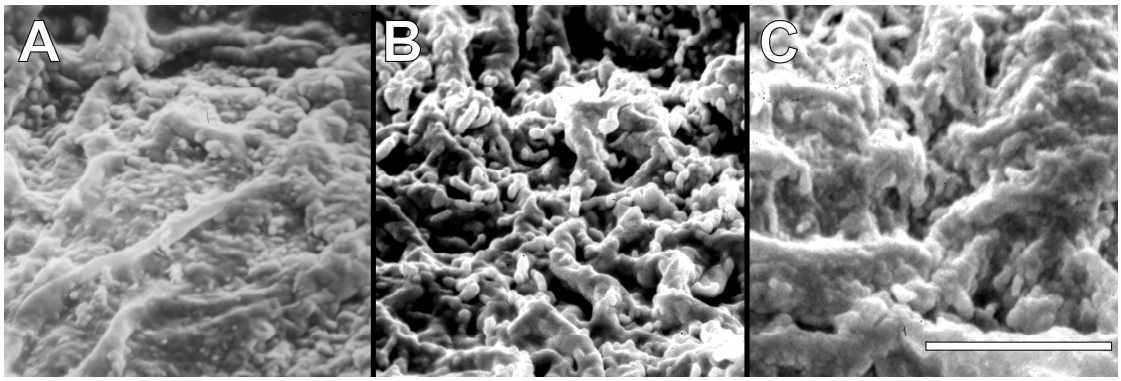
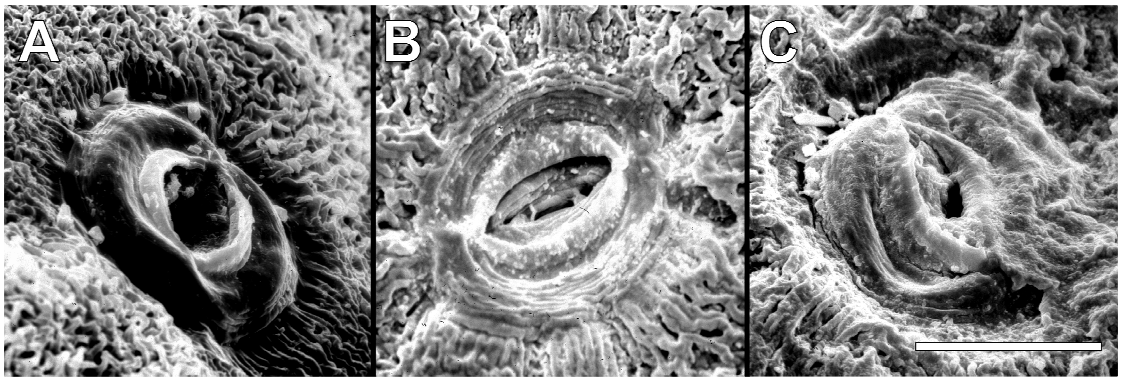

A
B
C

Figure 3.6. Images of developed and functioning stomata on berries of (A) *V. hybrid* 'Red Amber' (PI: 588296) at 8 days post bloom, (B) *V hybrid* 'Tom's Favorite' (PI: 483176) at 21 days post bloom and (C) *V. hybrid* 'Tom's Favorite' at 28 days post bloom. The last time point shown (C) shows a stoma covered with newer layers of cuticle. This deposition eventually covers the stoma leading to the formation of lenticels in mature berries [5, 22]. The scale bar represents 5 μm.


A
B
C

Besides OA, the cuticles were composed of a mixture of long-chain fatty acids, *n*-alkanes, aldehydes and primary fatty alcohols, whose concentrations varied depending on the genotype and developmental time point (Table 3.1).

These chemical classes did not vary greatly in amount at any time point within genotypes. This differs from previous studies looking at several *V. vinifera* varieties, where other than OA, the cuticle was composed chiefly of primary fatty alcohols [2, 5, 21]. Comménil *et al*, 1997 [5] also demonstrated that the fatty alcohol portion of the cuticle decreased during development, being replaced by the other chemical classes, which was not borne out in the 28-day timeframe of this study (Table 3.1).

Notable chain length bias was seen for all genotypes (Figure 3.1). There was a tendency for the chain length within a chemical class to shift toward longer-chained molecules as the clusters developed. This pattern was observed in the primary fatty alcohols where 26-carbon chain lengths predominated in the earlier time points and then shifted to 28-carbon chain lengths in the later. This does differ in the *V. rupestris* genotype (PI:588224), where the 24-carbon alcohols increase during development and the 26- and 28- carbon alcohols decrease. Other chemical groups showing a similar pattern include the aldehydes and alkanes (Figure 3.1).

When chain length preference was calculated regardless of developmental time point, 26-carbon fatty acids were preferred over other chain lengths in all three genotypes, while 30-carbon aldehydes were strongly preferred in the CR and SU genotypes, as well as 33-carbon alkanes (Table 3.2). Similar preferences are reported in a previous study of *V. vinifera* cuticle waxes [21]. In fact, the average chain length bias for all time points across all three genotypes had the same result (data not shown). However,

while there were similarities among the examined genotypes, each genotype did show a unique pattern of preferred molecule chain length (Figure 3.1).

Berry surface topology through development. Cuticle structure changed in all genotypes during development and was similar to structural development described in previous studies [3, 5, 22]. However, differences were observed among genotypes in the current study. The generalized pattern of cuticle development began with a highly organized array of cuticular, or vermicular [5], ridges that did not show a directional pattern but did impart a significant amount of three-dimensional structure (Figures 3.2A, 3.3A, 3.4A). These surfaces began to change at two weeks of development (Figures 3.2B, 3.3B, 3.4B). While cuticular ridges remained, they were spread further apart and interspersed by narrower and shallower minor ridges. The development in the two interspecific hybrids appeared to remain on a developmental path similar to that previously seen in *V. vinifera* [5], which resulted in continued flattening of the cuticle. By 28 DPB, the cuticles of these genotypes ends up nearly flat, with any remaining ridges covered in a smooth wax layer (Figures 3.2C-D, 3.3C-D). In contrast was development in the persistently susceptible *V. rupestris*, which retained a structure similar to that at 15 DPB, with both cuticular and minor ridges persisting (Figure 3.4C-D).

Rosenchrist and Morrison (1988) suggested that the grape berry cuticle loses its organized structure by being stretched during development [23], which could account for the continued presence but less dense pattern of cuticular ridges at 28 DPB (Figures 3.2D, 3.3D, 3.4D). Another possibility is that new layers of cuticular wax are deposited on top of the previous layers, in effect filling in the space between ridges, which could also account for the thickening observed in developing cuticles [7].

Table 3.2. Relative representation of each chain length within chemical class averaged across developmental time points, by *Vitis* genotype.

		(%)			
Chemical class	Carbon number	*V. hybrid* 'Tom's Favorite'	*V. hybrid* 'Tom's Favorite' shot berry	*V. hybrid* 'Red Amber'	*V. rupestris* 'R-65-44'
Acids	C16	0.2	4.5	1.2	1.9
	C18	0.6	2.7	0.7	9.0
	C20	0.9	16.9	3.4	6.5
	C22	3.3	9.6	15.4	8.0
	C24	12.9	7.5	11.3	30.1
	C26	63.4	43.6	56.0	27.1
	C28	11.8	12.4	10.5	10.6
	C30	6.8	2.8	1.6	6.8
Aldehydes	C22	1.8	7.0	3.5	10.2
	C24	2.0	9.7	6.8	8.4
	C26	9.2	9.2	41.7	16.4
	C28	48.6	37.5	34.6	14.7
	C30	38.4	36.5	13.4	50.2
1° Alcohols	C22	1.5	2.4	8.4	2.8
	C24	11.5	9.3	25.9	5.1
	C26	24.6	23.0	29.3	14.6
	C28	34.1	24.9	25.7	34.9
	C30	28.3	40.4	10.7	42.6
Alkanes	C21	1.5	2.2	1.1	1.9
	C23	1.1	5.2	4.6	5.7
	C25	9.5	11.1	5.1	9.8
	C27	15.6	15.8	16.3	13.5
	C29	18.2	17.5	33.7	18.0
	C31	25.0	18.0	33.0	22.9
	C33	29.2	30.2	6.2	28.0

This latter hypothesis is supported by the observed filling in of the stomata on the berry surface observed in the current study (Figure 3.6C). Deposition of new wax layers on top of existing cuticle is also evident in *V. hybrid* 'Red Amber' (Figure 3.3D). Regardless of deposition method, the cuticular waxes must accommodate the rapid and exponential increase in surface area that occurs during grape berry development.

Wax structure has previously been attributed to chemical composition [17]. Using SEM and thin layer chromatography, Comménil *et al.* [5] found changes in both the topology and chemistry of cuticles in the same timeframe as this study, which were suggested to be interrelated. However, while the cuticles observed in the current study underwent dramatic structural changes in the first four weeks of development, a similar level of chemical flux was not observed during the same time period (Figure 3.1).

A potential reason for this disparity is the possibility that the OA, which was found to comprise the majority of the cuticle wax at every time point, could reside at the cutin-epicuticular interface, and the other wax components at the berry surface. Radler and Horn [21] described the OA as "hard wax" and the other aliphatic components as "soft wax", which is easily removed by petroleum ether. This soft wax, which underwent a greater level of change in this study, could be responsible for the structural changes observed.

Fully formed stomata were observed on the surface of berries examined (Figure 3.6). Stomata on grape berry surfaces have been previously reported [5, 18, 22], and likely have function in active gas and water vapor transfer. As the berries mature, the

stomata became covered in wax deposits (Figure 3.6C) until they were no longer functional, ultimately becoming lenticels in mature berries [5, 22].

Shot berry chemistry and topology through development. The chemistry of the cuticle of a shot berry was drastically different from that of normally developing berries from the same cluster (Table 3.1). The most dramatic aspect of this difference was the relatively low amount of OA found at each time point of development, approximately one-third the amount found in the normally developing berries (Table 3.1). However, other aspects of cuticle chemistry were similar between shot berries and normally developing berries, including relative representation of other chemical classes and the temporal dynamics of chain length preference (Table 3.2, Figure 3.1). This suggests that differences established between normal and shot berries during the first few days of berry development have a major influence on the quantity of OA but neither on the ratio of other chemical classes incorporated into the cuticle nor on the chain lengths of each chemical class used.

The cuticle topology of the shot berries was not distinguishable from normally developing berries at the earliest time point, displaying the same cuticular ridges and three-dimensional structure (Figure 3.5). However, the cuticle did not continue to develop and retained the structure of a one-week old berry throughout the growing season. This retention is consistent with the view that shot berries are stunted in development, not progressing past the earliest structures formed. The similarity of the shot berry cuticle structure along with the aforementioned difference in chemistry further implies that the chemistry of the cuticle is not cleanly tied to the exterior structure.

Additionally, shot berries demonstrate that the smoothing of the berry surface seen in normally developing berries is not due solely to weathering. Shot berries maintain early cuticle topology throughout the growing season when exposed to weathering effects. This suggests developmental processes are the primary contributor to topographical development.

Development of shot berry cuticle morphology also points to the need for fertilization and active development for *V. hybrid* 'Tom's Favorite' to develop the constitutively resistant phenotype observed. It remains unknown if the observed differences in cuticle chemistry and morphology are responsible for the differences in the resistance phenotype and additional investigation is needed.

Cuticle development and ontogenic resistance. Using selected genotypes which exhibit distinct phenotypes (*i.e.* ontogenic resistant and persistently susceptible) allowed for the association of cuticle development to the relative powdery mildew susceptibility. It should be noted that the normally developed berries from *V. hybrid* 'Tom's Favorite' were not informative in regard to OR, as the mechanism by which this genotype exhibits CR is unknown. They primarily serve as a benchmark by which to evaluate the persistently susceptible shot berries from the same clusters.

Tetracosanal (C24 aldehyde) closely followed an expression pattern implicating its involvement in host resistance. The concentration decreased during development of *V. hybrid* 'Red Amber' while remaining elevated in both persistently susceptible tissues tested (*e.g. V. rupestris* 'R-65-44' and shot berries from *V. hybrid* 'Tom's Favorite') (Figure 3.1). Fatty acids as a group also were elevated in ontogenically resistant tissue relative to the susceptible tissues (Table 3.1). Having these few compounds from a

complex mixture of waxes suggests that ontogenic resistance is not controlled by a single wax compound, which has been suggested for other pathosystems (*e.g.* *Colletotrichum gloeosporioides*–Avacado, *Fusarium solani pisi*–pea) [16].

It is possible that compounds not identified also contribute to the transition from susceptible to resistant, such as fatty acid esters, which have been shown to be a constituent of berry cuticles [2, 5, 21]. In the current study, esters were not detectable due to their late elution time (>40 min) and the high baseline which resulted from the very large OA peak earlier in the elution order. However, this study did measure the predominant components of the grape berry cuticle as described above.

A correlation between gain of resistance and cuticle topology emerged. Two genotypes (*V. hybrid* 'Red Amber' and 'Tom's Favorite') had similar cuticle topological development to various genotypes of *V. vinifera* [3, 5, 22]. This commonly observed progression from cuticular ridges to flat wax platelets during the first month after fertilization thus appears to typify normal grape berry development. The susceptible *V. rupestris* diverges from this progression and exhibits a cuticle surface through the first month of development that approximates the early, susceptible developmental time points of OR genotypes. This suggests that powdery mildew requires for infection a cuticle exhibiting significant surface topology composed of cuticular and minor ridges. The aspects of this cuticular structure influencing the interactions between the host cuticle surface and the powdery mildew fungus are not clear and require additional study.

This investigation into the relation between grape berry cuticle chemistry and structure during the first month of berry development has shown that some aspects of chemistry

and structure are conserved for two of the genotypes considered while the persistently susceptible genotype diverges in cuticle structure and chemistry. This study was successful uncovering developmental patterns correlating to ontogenic resistance, although questions remain for fully explaining the role of the berry cuticle in the gain of resistance. The findings significantly contributed to our understanding of cuticle development and to the growing body of research into the molecular basis for ontogenic resistance to grapevine powdery mildew. This can serve as the foundation for additional research into the close interaction between the berry cuticle and the pathogenic fungus.

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FUTURE DIRECTIONS

This project began with the premise that screening for disease susceptibility on a diverse collection of *Vitis spp.* would yield phenotypic variation in ontogenic resistance (OR) sufficient enough for downstream, mechanistic studies. This screen was to build on the studies first describing this form of plant host resistance [4-6, 8, 9, 14]. The phenotypic variation discovered [10] was utilized for the studies presented in Chapters 2 and 3 of this text. While these two chapters explored different questions, they both shed new light on the understanding of OR.

Chapters 2 and 3 both uncovered patterns of development that correlate to the ontogenic gain of resistance. While there was no distinct pattern of protein expression relating to the development of the berry cuticle, the correlation of OR with aspects of normal berry development, such as photosynthesis and primary and secondary metabolism, suggests that routine developmental processes are involved in the cluster's ability to be resist infection. Three proteins negatively correlated to the development of OR are involved in lipid synthesis, suggesting that membranes or the cuticle could be in involved in OR. The potential role of the cuticle is also bolstered by the findings in Chapter 3 that the berries that exhibit resistance (either constitutively or ontogenically) have cuticles that develop topologically in a similar fashion to *V. vinifera*, which had been previously studied [2, 3, 13]. Conversely, the single genotype that remains susceptible has a cuticle which is distinct in its development, diverging from the other genotypes studied, as well as from *V. vinifera*. Taken together, these findings might explain why previous studies looking at aspects considered to be part of host defense did not fully uncover the basis for OR [4, 6].

The single, persistently susceptible genotype discovered (*V. rupestris* 'R-65-44') could be used for the generation of new genetic lines. These lines would be generated by crossing the susceptible *V. rupestris* with OR genotypes possessing desirable traits, such as the gibberellic acid (GA) insensitive dwarf grapevine *V. vinifera* 'Pixie' [1, 7] and its derivatives. If a GA insensitive grapevine population segregating for OR and persistently susceptible berries was obtained, it would be available for future investigation of OR.

To utilize these new genetic lines to their fullest potential, a significant amount of characterization needs to be carried out on the 'Pixie' genotype. We assume *V. vinifera* 'Pixie' will exhibit the same OR phenotype as all other *V. vinifera* genotypes investigated to date [1, 5, 9, 14], although this trait has yet to be assessed. Before segregation studies of new genetic lines can be conducted efficiently, the susceptibility period of *V. vinifera* 'Pixie' needs to be determined, which could be conducted using the detached berry method described in Chapter 1 [10]. Besides allowing full use of new genetic lines, determining the susceptibility of *V. vinifera* 'Pixie' will further develop this new grapevine as a research tool for studies of several viticultural questions beyond those pertaining to OR.

These new genetic lines could be used to confirm and further understand the protein expression patterns presented in Chapter 2. Having a genetically related population exhibiting variation in ontogenic resistance would ameliorate a major hurdle encountered during the analysis, namely the large and complex sample structure needed for the phenotypic bulking strategy. An experimental design using a segregating population would reduce the occurrence of diverged homologous proteins that fail to co-migrate in protein gels. This population in a bulked segregrant analysis

design would be more economical due to a reduced sample size. Additionally, regardless of the population used, the developmental time surrounding the of gain resistance could be more precisely targeted, allowing that fewer gels be run.

Potential exists for conducting reverse genetic studies to further explore hypotheses raised in this dissertation. For example, the role of proteasome activity can be studied by knocking out specific aspects of the proteasome catalytic system to search for effects on OR. This could be carried out by adapting techniques for virus-induced gene silencing (VIGS) mediated transformation presented in Muruganantham *et al.*, 2009 [11]. This technique uses *Agrobacterium*-mediated transformation with *Grapevine virus A* as a VIGS vector. This method targets propagated *V. vinifera* plantlets for transformation and gene activity knock out. Using dwarf grapevines would also aid this method for studying grape berry phenotypes due to their fast regeneration and fruiting time period [1]. Of course, this technique could be used to study individual gene influence, or to disrupt metabolic or biosynthetic pathways.

Working with genotypes that more amenable to molecular and biochemical research, such as 'Pixie', could also be an important asset for further investigation of the importance of the cuticle in ontogenic resistance and berry development. As reported in Chapter 3, both chemistry and topology were implicated in ontogenic resistance. Additional, more targeted studies could be conducted, using dwarf grape vines that exhibit OR and those that remain susceptible.

These studies could be successful in investigating the role of minor cuticle components not quantified in the presented work. These studies would benefit from lessons in sample extraction, preparation and analysis learned during the previous

work, which would provide higher resolution chemical data. This improved resolution would allow for the quantification of molecules such as fatty acid esters that elute after oleanolic acid, which were obscured by the high baseline encountered in the previous work. Lowering the sample concentration would also aid in chemical determination by keeping sample within the optimal concentration for gas chromatography. Additionally, being able to grow the vines in controlled conditions should also improve analysis resolution due to reducing the input of interfering compounds such as pesticides applied in the vineyard.

Also of interest is the development of a bioassay to investigate the role of candidate cuticular waxes discussed in Chapter 3, such as fatty acids and tetracosanal. A major hurdle to this type of bioassay is the ability to deliver the wax to the surface, as the introduction of solvent, necessary for keeping the wax in solution, will disrupt the existing cuticle. Assuming this issue can be resolved, coated berry surfaces could be assessed using the detached berry method outlined in Chapter 1 [10].

Additional studies into cuticle involvement in OR could be explored, including exploration of cuticle-embedded proteins [12], as well as investigating other cuticle development dynamics (*e.g.* cuticle deposition, cuticle tensile strength and elasticity, cutin and intercuticlular wax composition). These studies will not only provide further information on the cuticle's role in OR, but will further explore the development during this dynamic period in the berry's life.

During the field and cuticle studies, shot berries became a topic of interest for several reasons. It was observed that these berries typically express a persistently susceptible phenotype, even if the normally-developing berries of the same clusters exhibit

constitutive resistance [10]. This differentiation must occur very rapidly as the earliest cluster inoculations (4 DPB) would exhibit this difference. This suggests that factors responsible for triggering the formation of a shot berry occur within the first couple days following anthesis, if not before, and act in a very rapid manner. Studies aimed at using shot and normally developing berries from selected genotypes for structural, chemical and gene expression studies could help uncover factors responsible for shot berry formation.

An interesting comparison would be to track gene expression during berry development in shot and normally developing berries from selected genotypes, including *V. vinifera* varieties. This assessment could be carried out in several ways, including using the *Vitis vinifera* Genome Array from Affymetrix for a broad perspective or quantitative, reverse transcription PCR for targeted studies. Genes that show differential expression at the same developmental time points in the shot and normal berries will provide clues as to the aspects of developmental altered to result in a shot berry. Other studies, such as carbon partitioning in shot berries, difference in hormone production and concentration and percentages of shot berries that arise due to failed fertilization will shed light onto this undesirable aspect of viticulture.

An interesting aspect of development that arose during the protein project was the apparently high level of photosynthetic activity. It would be interesting to explore this aspect of early berry development further. Measuring rates of photosynthesis in berries during the first month of development would give clues as to whether or not the photosynthesis occurring is sufficient to contribute to later sugar accumulation. Scanning electron micrographs of early berries also showed apparently working stomata in young berries. Exploring early berry photosynthesis could also provide a

more complete picture as to the functionality of those stomata.

By further exploring the mechanisms of ontogenic resistance, we stand to gain a great deal of understanding about host resistance in this important fruit crop. This understanding can contribute to improvements in management practices, genotype development and environmental health.

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